



(43) International Publication Date
13 January 2005 (13.01.2005)

PCT

(10) International Publication Number
WO 2005/003384 A1

(51) International Patent Classification⁷: C12Q 1/68

(21) International Application Number:
PCT/DK2004/000480

(22) International Filing Date: 5 July 2004 (05.07.2004)

(25) Filing Language: English

(26) **Publication Language:** English

(30) Priority Data:

PA 2003 01018	3 July 2003 (03.07.2003)	DK
60/484,926	3 July 2003 (03.07.2003)	US

(71) **Applicant** (for all designated States except US): **DANMARKS OG GRØNLANDS GEOLOGISKE UNDERSØGELSE** [DK/DK]; Øster Voldgade 10, DK-1350 Copenhagen K (DK).

(72) Inventors: and

(75) **Inventors/Applicants (for US only):** BENDER, Mikkel [DK/DK]; Østerfarimagsgade 37, DK-2100 Copenhagen Ø (DK). JACOBSEN, Carsten, Suhr [DK/DK]; Annebergvej 17, DK-2700 Brønshøj (DK).

**(74) Agent: PLOUGMANN & VINGTOFT A/S; Sundkrogs-
gade 9, P.O. Box 831, DK-2100 Copenhagen Ø (DK).**

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

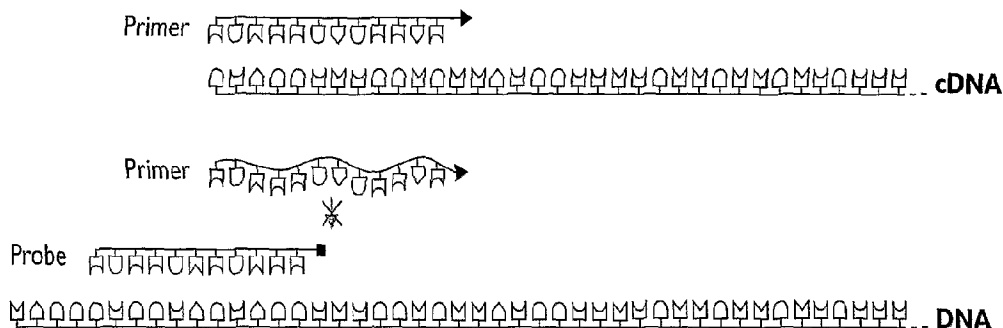
(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SI., SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHOD FOR SELECTIVE DETECTION OF A TARGET NUCLEIC ACID



(57) Abstract: The present invention pertains to methods and means for detecting the presence within a given environment of one or more target nucleic acid sequences while preventing or reducing interference from related nucleic acid species. In a primary aspect the invention provides a method for detecting the presence or absence of at least one target nucleic acid sequence in a sample that further contains a nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence, the method comprising the step of contacting the sample with at least one nucleic acid probe that is capable of selectively binding to a continuum of at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence. The invention may in particular be used in the detection of pathogenic organisms or as part of procedures for bioremediation of environmental contamination or pollution. Other aspects of the invention provides compositions and kits-of-parts to be used in the invention.

Method for selective detection of a target nucleic acid

FIELD OF INVENTION

- 5 The present invention relates to a method for detecting the presence within a given environment of one or more target nucleic acid sequences while preventing or reducing interference from related nucleic acid species. Being based on the use of blocking probes the method will be of use for instance in the detection of gene expression in general, and in particular in the detection of microbial and viral activity and as a diagnostic tool.
- 10 Furthermore the invention relates to compositions and kits of parts comprising components to be used in the method.

GENERAL BACKGROUND AND PRIOR ART

- 15 PCR and related technologies have become widely used for the detection of nucleic acid molecules present in biological systems. In particular, the RT-PCR technology has proven to be a powerful tool when analysing activity characterised by transcription of specific genes, such as microbial activity and responses to environmental challenges (4, 5, 6) as well as the initiation and the progression of pathological processes. However, the specific
- 20 nucleic acid molecules, which one aim at detecting, are often present at a very low amount in samples from biological systems and their detection is often complicated by the presence of molecular species having a structure related to that of the molecule to be detected. Such related molecular species may, even at a low amount, cause the generation of false results, either positive or negative. This clearly indicates the need for further
- 25 development of sensitive, specific and efficient procedures.

- For instance, when analysing low abundance RNA by RT-PCR procedures the elimination of contaminating DNA is of fundamental importance. Extraction of highly pure DNA-free RNA from biological samples usually presents a trade off between sample quality and sample
- 30 quantity and co-extracted DNA is often present in excess of recovered RNA (7). Removal of residual contaminating DNA is commonly achieved by treating samples with DNase. However, DNase treatment is not well suited in a number of applications. When the starting material is an environmental sample, for instance, humic acids and other organic compounds present in such samples have been shown to inhibit DNases, while RNases are
- 35 not inhibited (8), and the time involved in the DNase treatment could prove critical especially when dealing with highly labile mRNA. The ubiquitous presence of RNases and the nature of the RNA molecule, renders it highly susceptible to degradation and half-lives of these molecules typically range between seconds and a few minutes. Furthermore, some

DNases possess residual RNase activity and when inactivating the DNase enzyme by heating to 70°C in the presence of Mg^{2+} , a chemical degradation of RNA is induced (9).

In order to overcome these problems numerous techniques have been developed which, in general, have focused on eliminating the amplification of molecular species structurally related to the target molecule. As for RT-PCR, a common approach is to use primers directed at the poly-A-tail which is added to most eukaryotic mRNA thereby selectively targeting the mRNA and not the corresponding genomic DNA. While this approach is widely used it can not, however, be applied to all biological systems. Thus, unlike eukaryotic mRNA, a poly-A tail is not added to the 3' end of procaryotic mRNAs rendering this technique inapplicable to, for instance, detection of bacterial activity. Detection of bacterial activity is the object of numerous diagnostic procedures as well as procedures for evaluating the quality of materials such as foodstuffs.

Increased specificity of PCR and RT-PCR reactions has been obtained by the use of nucleotide probes, which selectively block the amplification of molecular species having a sequence related to that of the target nucleotide sequence. In general, nucleic analogues such as PNA have been preferred when designing such blocking probes. PNA bind tightly to complementary sequences of DNA and the thermal stability of PNA/DNA duplexes at physiological ionic strength is generally higher (per base pair) than that of the corresponding DNA/DNA duplex (10, 11). This unique property and the inability of PNA to function as a primer for DNA polymerase allows PNA to play the role of a potent sequence specific PCR blocking molecule. Probes consisting of PNA have previously been used successfully to eliminate PCR product formation in a sequence-specific manner (10, 12, 13, 14). However, all probes previously used in attempts to block specific PCR reactions have been placed within the sequence of interest, their use again being restricted to applications such as blocking the amplification of undesired sequences containing nucleotide polymorphism(s) or intronic sequences not present in the target sequence. In conclusion there is still an explicit need for an efficient technique for preventing the amplification of unwanted nucleic acid species, a technique which can be applied universally.

SUMMARY OF THE INVENTION

In the broadest sense, the present invention relates to a method for detecting the presence or absence of at least one target nucleic acid sequence in a sample that further contains a nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence, the method comprising the step of contacting the sample with at least one nucleic acid probe that is capable of selectively binding to a continuum of at least a part of

the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence.

In another aspect, the invention relates to a composition useful in the detection of the
5 presence or absence of at least one target nucleic acid sequence in a sample that contains a nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence, said composition comprising at least one nucleic acid probe that is capable of selectively binding to a continuum of at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule
10 adjacent to said corresponding sequence.

In a further aspect, the invention pertains to a kit for detecting the presence or absence of at least one target nucleic acid sequence in a sample that contains a nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence, said kit
15 comprising at least one nucleic acid probe that is capable of selectively binding to a continuum of at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence.

20 DEFINITIONS

As used herein, the term 'binding' refers to the association of molecules by intermolecular forces. In the present context binding of one nucleic acid to another refers to the interaction of two or more nucleic acids by base pairing, such as the formation of Watson-
25 Crick base pairs or by alternative forms of base pairing such as the formation of Hoogsteen base pairs.

In the present context the term 'conserved sequence' refers to a base sequence in a nucleic acid molecule or an amino acid sequence in a protein that has remained relatively
30 or essentially unchanged throughout evolution. The term may refer specifically to sequences, which are identical in one or more living species, such as one or more species within the same genus, family, order, subclass, class, subphylum, phylum, subkingdom, or kingdom.

35 The terms 'nucleic acid', 'nucleic acid molecule' and 'nucleic acid sequence' as used herein refer to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimics/mimetics thereof. This term includes molecules composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) phosphodiester bond linkages as well as molecules having non-naturally occurring nucleobases, sugars and

covalent internucleoside (backbone) linkages which function similarly or combinations thereof. Such modified or substituted nucleic acids are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases and other enzymes, and are in the present context described by the terms "nucleic acid analogues" or "nucleic acid mimics". Preferred examples of nucleic acid mimics/mimetics are peptide nucleic acid (PNA-), Locked Nucleic Acid (LNA-), xylo-LNA-, phosphorothioate-, 2'-methoxy-, 2'-methoxyethoxy-, morpholino- and phosphoramidate- containing molecules or the like.

10

The nucleic acid, nucleic acid molecule or nucleic acid sequence may, for instance, be composed entirely of deoxyribonucleotides, entirely of ribonucleotides, entirely of nucleic acid mimics or analogues or chimeric mixtures thereof. The monomers are typically linked by internucleotide phosphodiester bond linkages. Nucleic acids typically range in size from a few monomeric units, e.g., 5-40, when they are commonly referred to as oligonucleotides, to several thousands of monomeric units. Whenever a nucleic acid or a nucleic acid sequence is represented, it will be understood that the nucleotides are in 5' to 3' order from left to right and that "A" denotes deoxyadenosine, "C" denotes deoxycytidine, "G" denotes deoxyguanosine, and "T" denotes thymidine, unless otherwise noted.

20

In general, as well as in the present context, the term 'target nucleic acid sequence' refers to a nucleic acid sequence that is the subject of hybridisation with a complementary polynucleotide, e.g. a primer or probe. As used herein 'target nucleic acid sequence' may also mean a nucleic acid sequence or a part of a nucleic acid sequence the presence of which is to be detected. Furthermore, a 'target nucleic acid sequence' may be a nucleic acid sequence or part of a nucleic acid sequence, which is complementary to a nucleic acid sequence the presence of which is to be detected. The sequence can be composed of DNA, DNA digests, RNA, and analogues thereof including sequences of synthetic nucleic acids and combinations of the aforementioned. More specifically, the target nucleic acid sequence may be a nucleic acid sequence which has been generated by transcription and/or amplification *in vitro*; examples including complementary DNA (cDNA) and the first strand generated in strand specific PCR.

30

In general, as well as in the present context, the term 'molecule comprising a sequence corresponding to the target nucleic acid sequence' refers to any nucleic acid sequence that is not the target sequence but comprises a sequence resembling the target sequence. Examples of such sequences could be a nucleic acid fragment containing the target

35

sequence or a sequence resembling the target sequence within its overall sequence as well as at least one further nucleotide.

In many embodiments of the present invention it is contemplated that the target nucleic acid sequence is a single stranded nucleic acid molecule for which a complementary strand can be generated. The target nucleic acid molecule may, however, also be a double stranded nucleic acid molecule.

In the present context, 'probe' means a nucleic acid or nucleic acid analogue which is useful for annealing or binding to a nucleic acid molecule, which is related to a target nucleic acid molecule such as a nucleic acid molecule corresponding to the target nucleic acid sequence or part hereof.

As used herein, the term 'annealing' refers to the association of single stranded nucleotides to form a double stranded structure such that a nucleotide in one strand of the double stranded structure undergoes specific Watson-Crick base pairing with a nucleotide on the opposite strand. The term also comprehends the pairing of nucleoside analogues, such as deoxyinosine, nucleosides with 2-aminopurine bases, and the like, that may be incorporated into the primers and probes of the invention.

The term 'upstream sequence' refers to a nucleic acid sequence, which is located at a position, which is 5' to a particular point or region within a nucleic acid molecule. In particular the term 'upstream' refers to sequences located in the opposite direction of the transcription reaction.

In the present context 'complementary sequence' or 'complement' refers to nucleotide sequences which will anneal to a nucleic acid molecule of the invention under stringent conditions. The term "stringent conditions" refers to general conditions of high, weak or low stringency. The term "stringency" is well known in the art and is used in reference to the conditions (temperature, ionic strength and the presence of other compounds such as organic solvents) under which nucleic acid hybridisations are conducted. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences, as compared to conditions of "weak" or "low" stringency.

As an example, high stringency hybridisation conditions comprise (1) low ionic strength and high temperature for washing, such as 0.015 M NaCl/0.0015 M sodium citrate, pH 7.0 (0.1xSSC) with 0.1% sodium dodecyl sulfate (SDS) at 50°C; (2) hybridisation in 50% (vol/vol) formamide with 5 x Denhardt's solution (0.1% (wt/vol) highly purified bovine

serum albumin/0.1% (wt/vol) Ficoll/0.1% (wt/vol) polyvinylpyrrolidone), 50 mM sodium phosphate buffer at pH 6.5 and 5 x SSC at 42°C; or (3) hybridisation in 50% formamide, 5 x SSC, 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5 x Denhardt's solution, sonicated salmon sperm DNA (50 µg/ml), 0.1% SDS, and 10% dextran sulfate at 5 42°C with washes at 42°C in 0.2 x SSC and 0.1% SDS.

In the present context and when referring to nucleic acids or nucleic acid sequences derived from other nucleic acids or nucleic acid sequences, the term "derived" specifies that the nucleic acid or nucleic acid sequences are generated, wholly or in part, by 10 polymerisation in vivo or in vitro of nucleosides using said other nucleic acid or nucleic acid sequence or part hereof as a template. Non-limiting examples of derived nucleic acids and derived nucleic acid sequences are sequences generated by DNA replication, transcription or reverse transcription, such as member of any of the functional classes of DNA and RNA sequences defined in the following detailed description of the invention. In particular, the 15 terms "derived nucleic acid" and "derived nucleic acid sequence" encompass ribosomal RNA (rRNA) and messenger RNA (mRNA) sequences as well as sequences of complementary DNA (cDNA) and DNA sequences generated by in vitro polymerisation.

When referring to a nucleic acid molecules 'corresponding' to for instance the target nucleic 20 acid sequence it is meant that this nucleic acid molecule or at least a part thereof is identical or has a certain degree of sequence identity to the target nucleic acid molecule or part thereof. The nucleic acid molecule or part thereof may thus be from 1-100%, such as at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, 25 at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100% identical to, for instance, the target nucleic acid molecule or part thereof.

When referring to 'identical sequences' herein, again it is meant sequences having a 30 certain degree of sequence identity. The sequences may thus be from 1-100%, such as at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100% identical.

35

The term 'sequence identity' indicates a quantitative measure of the degree of homology between two amino acid sequences or between two nucleic acid sequences of equal length. If the two sequences to be compared are not of equal length they must be aligned to give the best possible fit, allowing the insertion of gaps or, alternatively, truncation at the ends

of the polypeptide sequences or nucleotide sequences. The sequence identity can be calculated as $\frac{(N_{ref}-N_{dif})100}{N_{ref}}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC ($N_{dif}=2$ and $N_{ref}=8$). A gap is counted as non-identity of the specific residue(s), i.e. the DNA sequence AGTGTC will have a sequence identity of 75% with the DNA sequence AGTCAGTC ($N_{dif}=2$ and $N_{ref}=8$).

In all polypeptide or amino acid based embodiments of the invention the percentage of sequence identity between one or more sequences is based on alignment of the respective sequences as performed by clustalW software (<http://www.ebi.ac.uk/clustalW/index.html>) using the default settings of the program. With respect to the nucleotide-based embodiments of the invention, the percentage of sequence identity between one or more sequences is also based on alignments using the clustalW software with default settings. For nucleotide sequence alignments these settings are: Alignment=3Dfull, Gap Open 10.00, Gap Ext. 0.20, Gap separation Dist. 4, DNA weight matrix: identity (IUB).

In the present context, by 'amplification reaction' is meant a reaction that produces one or more copies of a sequence of nucleic acids by repeated extension of a probe or primer.

'Extension' may occur by virtue of polymerisation of individual nucleotide monomers, as in PCR, or it may occur by the addition of prefabricated oligonucleotide segments, as in LCR, or by a combination of these as in gap LCR or Repair Chain Reaction (RCR). Though not essential to the invention, ideally the extension reactions are performed repeatedly and the extension products themselves may serve as templates to produce an exponential generation of products.

' T_m ' can be defined as the temperature at which 50% of a nucleic acid and its perfect complement are in duplex. The denaturation of double stranded nucleic acids causes a shift in the absorbance of UV light at 260 nm wavelength, an effect which can be assayed by determining the optical density at 260 nm (OD_{260}). T_m is defined as the temperature corresponding to 50% denaturation, i.e. where the (OD_{260}) is midway between the value expected for double stranded nucleic acids and the value expected for single stranded nucleic acids. The T_m of perfectly complementary duplexes can be calculated as follows:

$$\begin{aligned} \text{DNA:} & \quad T_m = 81.5 + 16.6(\log_{10}[\text{Na}^+]) + 0.41(\%GC) - 500/\text{length} \\ \text{RNA; RNA-DNA:} & \quad T_m = 79.8 + 18.5(\log_{10}[\text{Na}^+]) + 0.58(\%GC) + 11.8(\%GC)^2 - 820/\text{length} \end{aligned}$$

Oligonucleotides: $T_m = 2(\text{no. of AT pairs}) + 4(\text{no. of GC pairs})$

As used herein, 'nucleic acid analogue' is understood to mean a structural analogue of DNA or RNA, designed to hybridise to complementary nucleic acid sequences (1). Through
5 modification of the internucleotide linkage(s), the sugar, and/or the nucleobase, nucleic acid analogues may attain any or all of the following desired properties: 1) optimised hybridisation specificity or affinity, 2) nuclease resistance, 3) chemical stability, 4) solubility, 5) membrane-permeability, and 6) ease or low costs of synthesis and purification. Examples of nucleic acid analogues include, but are not limited to, peptide
10 nucleic acids (PNA), locked nucleic acids "LNA", 2'-O-methyl nucleic acids, 2'-fluoro nucleic acids, phosphorothioates, and metal phosphonates. Nucleic acid analogues are described in (2) and (3).

As used herein, the term 'mRNA' means the presently known mRNA transcript(s) of a
15 gene, and any further transcripts, which may be identified.

As used herein, the term 'gene' means the gene including exons, introns, non-coding 5'- and 3'-regions and regulatory elements and all currently known variants thereof and any further variants, which may be elucidated.

20

DETAILED DESCRIPTION OF THE INVENTION

The object of the present invention is to provide an efficient method for detecting the presence within a given environment of one or more target nucleic acid sequences while
25 preventing or reducing interference from related nucleic acid species such as the species defined herein. The method is based on the use of probes, which are capable of binding to the related nucleic acid species thereby preventing their detection.

Thus, in a broad sense the invention relates in a first aspect to a method for detecting the
30 presence or absence of at least one target nucleic acid sequence in a sample that further contains a nucleic acid molecule which is related to the target nucleic acid molecule. More specifically this related nucleic acid molecule may comprise a sequence corresponding to the target nucleic acid sequence. The method comprises the step of contacting the sample with at least one nucleic acid probe that is capable of selectively binding to a continuum of
35 at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence. It is to be understood that the method according to the invention may be applied not only when the main objective is to detect the presence of a target nucleic acid but also for any purpose involving a step of detecting the presence or determining the amount of one or

more given nucleic acids. It is further to be understood that 'a sequence corresponding to the target nucleic acid molecule' may be identical or complementary to the target nucleic acid sequence or a part hereof.

- 5 Inherent in the method of detecting the presence or absence of target nucleic acid sequences may be one or more steps of increasing the amount of target nucleic acids until reaching levels at which detection becomes possible. A crucial feature of the method may therefore be the presence of one or more extendable oligonucleotide primers. Whether occurring naturally as in a purified restriction digest or produced synthetically such primers
- 10 should be able to act as a point of initiation of synthesis when placed under conditions in which a primer extension product complementary to a target sequence is induced. Therefore the method may further comprise contacting the sample with a first extendable primer.
- 15 According to this aspect of the invention annealing of at least one nucleic acid probe to the molecule comprising a sequence corresponding to the target nucleic acid sequence will prevent annealing of the extendable primer hereto and/or extension of the primer. Under the same conditions, however, the blocking probe will not affect attachment or annealing of the extendable primer to the target nucleic acid sequence. The blocking probe will
- 20 thereby prevent PCR amplification of the molecule comprising a sequence corresponding to the target nucleic acid, without affecting amplification of the target nucleic acid itself.

It is envisaged that the target nucleic acid molecules have one or two defined ends and that the related nucleic acid molecules continue beyond these points. Therefore, the

25 sequence-specific blocking probes can be designed, targeting regions of the related nucleic acid molecules immediately upstream of and proceeding through the sequences corresponding to a defined end of the target nucleic acid sequences. In RT-PCR, the one or two ends of the target nucleotide will correspond to either the RNA 5' end at the transcription initiation site or a RNA 5' end generated by RNA processing and one

30 generated by the primer used in the RT-procedure. The corresponding chromosomal DNA sequence, on the other hand, will extend beyond the points corresponding to the RNA termini. Accordingly, in one embodiment of the invention the continuum of at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence comprises a

35 transcription initiation site and its upstream sequence.

Alternatively, the continuum of at least a part of the nucleic acid molecule corresponds to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence comprises a site containing the sequence targeted by an

extendable primer and its upstream sequence. This may be the case for instance in strand specific-PCR, where one end of the target nucleotide will correspond to the end generated by the primer used in the first amplification cycle in a PCR-procedure. The corresponding complementary nucleic acid sequence, on the other hand, will extend beyond this point
5 and act as target for blocking probes added prior to subsequent amplification cycles.

According to the main object of the invention hybridisation of the nucleic acid probe to the nucleic acid molecule corresponding to the target nucleic acid sequence reduces, hinders or prevents annealing of the at least one extendable primer hereto. This results in a reaction
10 in which detection and/or amplification of the target nucleic acid sequence will prevail over detection and/or amplification of the nucleic acid molecule corresponding to the target nucleic acid sequence. Therefore, it is preferred that the first extendable primer and a part of the nucleic acid probe are both complementary to a 3' region within said part of the nucleic acid molecule corresponding to the target nucleic acid sequence. By a 3' prime
15 region is meant a region in proximity of the 3' end of the given nucleic acid sequence

While the extendable primer and a part of the nucleic acid probe must be complementary to a suitable sequence within the target nucleic acid molecule this is to be interpreted so as to mean that the primer and probe must be sufficiently complementary to ensure
20 annealing to their target sequences under stringent conditions. It is conceivable, however, that one may gain advantages by using mismatched or degenerate primers. One advantage could be, for instance, in a PCR reaction, the possibility of altering the melting temperature (T_m) of the (undesired) duplex between the blocking probe and the amplified target sequence by introducing mismatches in its sequence. Additionally the target
25 sequence may contain degenerate positions within the primer target sequence requiring the use of degenerate primers for amplification. In particular, mismatched or degenerate primers may be used to target different, but closely related nucleic acid sequences, such as mRNAs from several closely related sources. Furthermore, such mismatches can be introduced in order to reduce the likelihood of the primer forming undesired secondary
30 structures. It may therefore be preferred that the extendable primer has at least one mismatch relative to its complementary sequence in the target nucleic acid sequence. Alternatively, divergence may be generated by incorporating a tag sequence into the extendable primer. Since the tag should not prevent extension of the primer it is preferably located away from the 5' end of the primer, more preferably it is a 3' tag
35 sequence. Addition of a tag sequence may further facilitate the use of higher primer annealing temperatures during the primer annealing step in PCR reactions, and eventually performing the probe annealing step and primer annealing step in a amplification reaction according to the present invention at more even temperatures, or even at the same temperature. The length of the mismatch or the 3' tag sequence may be from 1 - 20

nucleotides, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 nucleic acids.

As mentioned, the nucleic acid probe must meet the dual criteria of being able to bind to
5 the nucleic acid molecule corresponding to the target nucleic acid sequence under the desired conditions while not being able to bind to the target nucleic acid sequence. Therefore, the probe may be composed of two distinct parts, a first part, which is complementary to a region within the nucleic acid molecule corresponding to the target
10 nucleic acid sequence, and a second part, which is complementary to a sequence of the nucleic acid molecule. Preferably, the first part of the probe is complementary to a 3' region within said part of the nucleic acid molecule corresponding to the target nucleic acid sequence, and a second part, which is complementary to a neighbouring sequence of the nucleic acid molecule.

15 When preparing nucleic acid probes and the extendable primers to be used in the present invention one will aim at designing probes and primers having favourable T_m -values. While, in general, longer probes and primers have a higher T_m they also hybridise with lower specificity at a given temperature as compared to shorter probes and primers. Therefore, the optimal length of the primers and probes is determined, at least in part, by
20 the nucleotide sequence at which they are directed and may therefore vary within and between the different embodiment of the invention. Accordingly, the probes and primers of the invention may be more than or equal to any length between 3 and 200 nucleotides, such as more than or equal to 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19,
25 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200 nucleotides in length.

30 In some preferred embodiments of the invention, the length of the nucleic acid probes is from 11 to 35 nucleotides in length, whereas the first extendable primers are from 11 to 27 nucleotides in length. In other preferred embodiments the length of the nucleic acid probe is from 8 to 20 nucleotides in length, whereas the first extendable primer is from 11 to 27 nucleotides in length.

35

As mentioned, the nucleotide probe may have two distinct parts, a first part, which is complementary to a region within the nucleic acid molecule corresponding to the target nucleic acid sequence and a second part, which is complementary to a sequence of the nucleic acid molecule. Considerations similar to the above apply when determining the

desired length of these parts. While the first part of the probe may constitute any proportion of the total length of the probe, the first part of the nucleic acid probe and the first extendable primer may be of equal length. This means that the first part of the nucleic acid probe and the first extendable primer are directed at the same 3' region within the
5 target nucleic acid.

Ideally, in order to prevent annealing of the nucleic acid probe to the target nucleic acid, the hybrid of the target nucleic acid and the extendable primer should have a higher T_m -value than the putative hybrid between the nucleic acid probe and the target nucleic acid.
10 It may be therefore be preferred that the first part of the nucleic acid probe is shorter than the first extendable primer.

Also, in a preferred embodiment of the invention, the T_m -value for the expected hybrid of the nucleic acid probe and the related nucleic acid molecule is higher than the T_m -value for
15 the expected hybrid of the first extendable primer and the target nucleic acid sequence. This is to assure that annealing of the nucleic acid probe to the related nucleic acid molecule will take place at a temperature at which annealing of the first extendable primer does not occur. Thereby, annealing of the nucleic acid probe to the related nucleic acid sequence is favoured over annealing of the first extendable primer to its complementary
20 sequence in the related nucleic acid molecule. Preferably the T_m value for the expected hybrid of the nucleic acid probe and the related nucleic acid molecule is from 1 to 20°C, such as from 2 to 15°C, from 5 to 12°C or from 8 to 10°C higher than higher than the T_m -value for the expected hybrid of the first extendable primer and the target nucleic acid sequence.

25 Other approaches of assuring that the nucleic acid probe anneals to the related nucleic acid molecule prior to annealing of the extendable primers include the inclusion of two annealing steps. This will allow annealing of the nucleic acid probe and annealing of the first extendable primer to occur in two successive steps. In a preferred embodiment the
30 step in which annealing of the nucleic acid probe occurs precedes the step in which annealing of the first extendable primer occurs. In order to obtain this sequential annealing a pre-primer annealing step may be introduced at a temperature, which is sufficiently high to only allow annealing of the probe. A subsequent lowering of the temperature allows the primer to anneal. In order to further promote annealing of the nucleic acid probe prior to
35 annealing of the first extendable primer it is preferred that the nucleic acid probe is present in molar excess as compared to the primer.

In addition, it may be preferred that the sample containing the target nucleic acid sequence is contacted with the nucleic acid probe prior to contacting it with any one of the

other components to be added, including extendable primers, nucleotides, enzymes, and buffers. Also, the sample containing the target nucleic acid sequence may preferably be contacted with the nucleic acid probe prior to any reverse transcription of nucleic acid molecules present in the sample or subsequent to such reverse transcription but prior to
5 any amplification of the transcribed nucleic acid species. However, these are just examples of methods, which can be employed in order, for instance, to favour annealing of the nucleic acid probe over annealing of the first extendable primer, and the present invention is not limited to these methods.

- 10 In theory the nucleic acid blocking probe used in the present invention may be any nucleic acid molecule that is not (naturally or modified such) extended by the action of the used DNA polymerase. Examples of such blocking probes are probes (some of which require a 5' modification) comprising or consisting of DNA, RNA, peptide nucleic acid (PNA-), Locked Nucleic Acid (LNA-), Intercalating nucleic acids (INA), xylo-LNA-, phosphorothioate-, 2'-
15 methoxy-, 2'-methoxyethoxy-, morpholino- and phosphoramidate- containing molecules or the like.

In a preferred embodiment of the present invention the nucleic acid probe is a nucleic acid analogue. In addition, it is preferred that the nucleic acid probe is a peptide nucleic acid
20 (PNA). Peptide nucleic acids are compounds that in certain respects are similar to oligonucleotides and their analogues and thus may mimic DNA and RNA. In PNA, the deoxyribose backbone of oligonucleotides has been replaced by a pseudo-peptide backbone. Each subunit, or monomer, has a naturally occurring or non-naturally occurring nucleobase attached to this backbone. One such backbone is constructed of repeating units of N- (2-
25 aminoethyl) glycine linked through amide bonds. PNA hybridises with complementary nucleic acids through Watson and Crick base pairing and helix formation. The Pseudo-peptide backbone provides superior hybridization properties, resistance to enzymatic degradation and access to a variety of chemical modifications.

- 30 PNA binds both DNA and RNA to form PNA/DNA or PNA/RNA duplexes, respectively. The resulting PNA/DNA or PNA/RNA duplexes are bound with greater affinity than corresponding DNA/DNA or DNA/RNA duplexes as determined by their T_m 's. In addition to increased affinity, PNA has also been shown to bind to DNA with increased specificity.

- 35 It is further envisaged that in certain applications the nucleic acid probe is preferably a locked nucleic acid (LNA). Structurally similar to RNA, LNA monomers are bicyclic compounds, which bear a methylene linker that connects the nucleotide sugar ring's 2'-oxygen to its 4'-carbon. Like PNA, LNA polymers obey standard base-pairing rules while offering greater stability and binding affinity to target nucleotide sequences.

As stated above the nucleic acid probe has a first part, which is complementary to a 3' region within the target nucleic acid. This is intended to mean that the nucleic acid probe will be able to hybridise to a 3' region within the target nucleic acid under stringent
5 conditions c.f. the definition given above. Again, this means that the nucleic acid probe does not need to be an exact match of the sequence at which it is directed and it may in certain instances be preferred that the nucleic acid probe has at least one mismatch, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20 or more mismatch(es), in its first part relative to its complementary sequence in the target nucleic acid sequence. One advantage could be, for
10 instance, in a PCR reaction, the possibility of altering the melting temperature (T_m) of the (undesired) duplex between the blocking probe and the amplified target sequence by introducing mismatches in its sequence. In addition such mismatches can be introduced in order to reduce the likelihood of the probe forming undesired secondary structures. Additionally the first and/or second part of the probe may contain one or more mismatched
15 or degenerate positions relative to its target sequence. Furthermore, the advantage could be, for instance, in a PCR reaction, the possibility of targeting degenerate positions within the probe target sequence. Another advantage could be related to the altering of the secondary structure of the probe.

20 The target nucleic acid sequence, the presence of which is to be detected using the method of the invention, may be virtually any type of nucleic acid molecule. According to the main aspect of the invention, however, the target nucleic acid sequence is a nucleic acid for which one or more complementary or partly complementary sequences and/or one or more identical or partly identical sequences exist. In particular, the target nucleic acid
25 sequence may be one of two complementary nucleic acid sequences. More specifically, the target nucleic acid sequence is one of either strand of a double stranded nucleic acid sequence or one of either strand of a potentially double stranded nucleic acid sequence.

In particular, the target nucleic acid sequence may be an RNA or cDNA sequence to which
30 an identical and/or complementary nucleic acid sequence exists. Also, the target nucleic acid sequence may be an RNA or cDNA sequence to which an identical double stranded nucleic acid sequence exists.

Several applications of the method according to the present invention may be devised in
35 which the target nucleic acid sequence is a DNA sequence. Several main classes of DNA exist, both when classified by amount and by function. The DNA sequence may thus be a unique sequence (single copy, low copy, nonrepetitive DNA) which is present as one or a very few copies per genome. This is typically the case for most genes, including introns, regulatory sequences and other DNA of unknown function. Alternatively, the DNA may be a

moderately repetitive DNA, which generally defines sequences present 10 - 10,000 copies per genome and include generally dispersed repeats corresponding to highly conserved multigene families (functional genes and pseudogenes) and transposable elements. Finally the DNA may be a highly repetitive DNA, which is defined as sequences present 100,000 to 5 1,000,000 copies per genome and normally found as tandem repeats although some superabundant (dispersed) transposable elements also fall into this class.

When classified by function one class of genomic DNA is genic DNA comprising DNA, which is actually expressed. Genic DNA may further be classified as mRNA (protein encoding 10 DNA), rDNA, tDNA, snDNA, etc. representing the different classes of gene product. A second class of DNA is regulatory DNA comprising DNA whose role is in the regulation of gene expression (e.g. promoters and enhancers) or the regulation of DNA function (e.g. origins of replication, matrix associated regions). Another class of DNA is intergenic DNA or spacer DNA which comprises introns and the DNA, which separates the genes from each 15 other. Satellite DNA is highly repetitive DNA found near centromeres, telomeres and at other sites, wherein some satellite DNA may play a role in chromosome function. Selfish DNA, which forms another functional class of DNA, is DNA whose role appears to be to mediate its own replication and survival within the genome, e.g. some satellite DNA, and transposable elements. Finally, DNA may be so-called junk DNA having no assigned 20 function.

According to a preferred embodiment of the invention, however the target nucleic acid sequence is an RNA sequence. As for DNA several classes of RNA exist as summarised in the following. Major classes of RNA are thus messenger RNA (mRNA), heterogeneous 25 nuclear RNA (hnRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA). Whereas some mRNA-like transcripts are untranslated mRNA it is in general transcribed from protein encoding genes and carries the message for translation. hnRNA refers to pre-spliced RNA, the unmodified transcripts of eukaryotic genes so called because of its great diversity of size compared to tRNA and mRNA.

30

Several minor classes of RNA exists such as telomerase RNA, guide RNA (gRNA), antisense RNA (mRNA-interfering complementary RNA, micRNA), ribozymes, initiator RNA (iRNA), small nuclear RNA (snRNA) or uridine-rich RNA (U-RNA), small nucleolar RNA (snoRNA), and small cytoplasmic RNA (scRNA). It is envisaged that any class of nucleic acid 35 comprising but not limited to those mentioned may constitute a target nucleic acid to be detected in a particular application of the method of the invention.

In a further preferred embodiment of the present invention the target nucleic acid sequence is derived from an RNA. Comprised hereby are target nucleic acids, which are

synthesised on the basis of a RNA as well as RNAs, which have been modified in any ways, and RNAs which are associated with other molecular species. Primarily, though, the target nucleic acid is synthesised by reverse transcription of an RNA belonging to any class of RNA including those mentioned above either *in vitro* or *in vivo* in an intact biological system. Accordingly, in this preferred embodiment the target nucleic acid sequence may be a cDNA sequence, such as the sequence of either strand of a double stranded cDNA. In a double stranded cDNA a first strand is generated by reverse transcription of an RNA, whereas the second strand is formed as the complementary strand by a DNA polymerase driven reaction using the first strand as a template.

10

The primary sequence of ribosomal RNAs vary considerably between species and a method, which is currently used for determining the presence of various organisms, is based on the amplification and detection of various parts of the ribosomal RNA from the organisms. In a preferred embodiment of the invention, therefore, the target nucleic acid sequence is derived from a ribosomal RNA.

15

Ribosomes are generally composed of a small and a large subunit. In *E. coli*, for instance, the small 30S subunit contains, besides proteins, a 16S rRNA whereas the large 50S subunit contains the 23S and 5S rRNAs. Eucaryotic ribosomes are larger and the small 40S subunit contains an 18S rRNA whilst the large 60S subunit contains three RNAs of 28S, 5.8S and 5S. Archaeal ribosomes resemble those of bacteria but some genera contain additional subunits similar to those of eukaryotes. In a currently preferred embodiment of the invention the target nucleic acid sequence is derived from 16S ribosomal RNA. It is envisaged that the method may employ the use of universal rRNA-directed primers and probes for the purpose of detecting the presence of any organism or it may use primers and probes which are specific for rRNA from a given species.

25

In another preferred embodiment of the present invention the target nucleic acid is derived from a messenger RNA. It will be understood that the method may be applied to the detection of abundant messengers as well as rare messengers for which it is particularly useful due to its high sensitivity. In particular, it may be preferred that the target nucleic acid is derived from a mRNA which does not possess a poly(A)-tail, such as a mRNA from a prokaryotic species.

30

In particular applications of the method according to the invention, the target nucleic acid is identical to a nucleic acid sequence, which is specific for one or more microbial species, or its complementary nucleic acid sequence. It follows that the method can then be used in the detection and/or quantification of these one or more microbial species in a given environment.

35

In particular applications it may also be preferred that the target nucleic acid is identical to a nucleic acid sequence, which is conserved among several microbial species, or its complementary nucleic acid sequence.

5

In currently preferred embodiments of the invention use of the method is directed at determining the presence of a pathogen or a pathogenic species within a given environment. In these embodiments the microbial species may comprise one or more pathogenic species.

10

The above mentioned characteristics of the target nucleic acid sequence may alternatively apply to part of the sequence only, such as the part to which said first and/or said second extendable primer is capable of annealing. Alternatively expressed, the first and/or second extendable primer may be complementary to a nucleic acid sequence, which is specific for one or more microbial species, or identical to a sequence, which is complementary to said specific nucleic acid sequence. The first and/or second extendable primer may likewise be complementary to a nucleic acid sequence, which is conserved among several microbial species, or identical to a nucleic acid sequence complementary said conserved nucleic acid sequence

20

In a prominent embodiment of the present invention the target nucleic acid sequence is derived from a bacterial messenger RNA. Inherent in this embodiment is use of the method of the invention in order to evaluate the presence of bacteria in different environments, such as in different biological systems.

25 In a currently most preferred embodiment of the invention, the target nucleic acid sequence is identical to a nucleic acid sequence which is derived from the *invA* gene of *Salmonella Spp.* or identical to the complementary sequence of said derived sequence. In particular the target nucleic acid may be identical to a nucleic acid sequence derived from an mRNA of the *invA* gene, such as a corresponding cDNA, or to the complementary sequence of said derived sequence. The following sequences exemplifies probes and primers and primers useful for this application:

Probe: H-AGA-CGA/C-CTG-GTA-CT-NH₂
Forward primer: 5'ACA GTG CTC GTT TAC GAC-3'
35 Reverse primer: 5'-ACT GGT ACT GAT CGA TAA-3'

Other embodiments of the invention pertain to detection, quantification and/or characterisation of components of biotic processes, hereunder components involved in the degradation or conversion of certain molecular species. In these embodiments, the target

nucleic acid may be identical to a nucleic acid sequence derived from a gene, the product of which is involved in the degradation or conversion of an undesired molecular species, or identical to the complementary sequence of said derived sequence. In particular the target nucleic acid may be identical to a nucleic acid sequence derived from an mRNA of said
 5 gene, such as a corresponding cDNA, or to the complementary sequence of said derived sequence. It is contemplated that the method according to the invention may be applied within the realm of bioremediation, that is in the prevention or reduction of environmental contaminations using for instance micro-organisms capable of degrading or converting the contaminants.

10

Accordingly, it may be preferred that said gene product is involved in the degradation or conversion of one or more pesticides. In a preferred embodiment of the invention illustrating this concept, the target sequence is identical to a nucleic acid sequence derived from the *tfdA* gene of *Ralstonia eutrophus* JMP134 or a part of hereof, such as a
 15 corresponding cDNA, or identical to the complementary sequence of said derived sequence. The *tfdA* gene product is critical in the degradation of certain pesticides MCPA and 2,4-D. The following exemplifies primer and probe sequences which can be applied when analysing *tfdA* gene expression.

20 Probe: Ac-CGT-TCC-AGC-GAT-GC

Forward primer: 5'AC(GC)-GAG-TTC-TG(CT)-GA(CT)-ATG-3'

Reverse primer: 5'CAG-GAT-GCC-GGT-ACA-CGA- 3'

The *tfdA* extendable primers have been designed for the combined targeting of several
 25 *tfdA* genes. As an alternative to the above sequence, the reverse primer sequence may be: 5'AGC-GAT-GCC-GGT-ACA-CGA- 3' , representing, according to "The Entrez Nucleotides Data Base", a perfect match with the published *tfdA* sequence from *Ralstonia eutrophus* JMP134. The use of degenerate primers or primers carrying a 3' tag sequence has been described in the preceding text as general embodiments of the invention.

30

For a number of other applications and combinations of target nucleic acid and nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence particular sets of extendable primers and probes may be devised. Examples of such sets of primers and probes are listed in table 1. Primers are designed on the basis of sequences
 35 from the "The Entrez Nucleotides database" which is a collection of sequences from several sources, including GenBank, RefSeq, and PDB, as well as primers shown in the "European ribosomal RNA database" (referred to as ribosomal database project).

Table 1.

Specific bacterial 16S and 23S		
Target nucleic acid	Primer	Probe
<i>Salmonella</i> 16S	TATTAACCACAACACCT-3'	AATTGCTGCGGTTATTAA
<i>Yersenia sp.</i> 16S	CCGATGGCGTGAGGCCC	CTGGGCACATCCGAT
<i>Campylobacter sp.</i> 16S	CATTGTAGCACGTGTGTC	TTGTATATGCCATTGTAGCA
<i>Clostridium perfringens</i> 16S:	GGGTACCGTCATTATCTTCCC	GGCTTCCTCCTTGGGTAC
<i>Legionella sp.</i> 16S	CCAACAGYWAGTTGACATCG T	TTTCATATAACCAACAGYWA G
<i>Listeria monocytogenes</i> 16S:	ATAGTTTTATGGGATTAGC	ACTGAGAATAGTTTTAT
<i>Mycobacterium Sp.</i> 16S:	GCCCGTATCGCCCGCACGCT CACA	CTCTAGTCTGCCCGTATC
<i>Vibrio vulnificus</i> 23S	TACTTGTAACCCATC	AAGATACTTGTA

Universal bacterial 16S		
Target nucleic acid	Primer	Probe
Universal Bacterial 16S	GCTGGCACGGAG	GCGGCTGCTGG
Based on primer BSR1541/20 (ribosomal database project)	TGATCCAGCCGCA	GAGGTGATCC
Based on primer BSR1407/16 (ribosomal database project)	GCGGTGTGTRC	GACGGGCGGT
Based on primer BSR1114/16 (ribosomal database project)	TGCGCTCGTTRC	GGGTTGCGC
Based on primer BSR798/15 (ribosomal database project)	GTATCTAATCCC	GGGGTATCTA
Based on primer BSR65/17 (ribosomal database project)	CTTGCATGTRTTA	TCGACTTGCATG
Based on primer	CTGCCTYCCGTA	CTGCTGCCT

BSR357/15 (ribosomal database project)		
Based on primer BSR534/18 (ribosomal database project)	CCGCGGCTGCTGGC	ATTACCGCG

Universal eukaryote nuclear 18S		
Target nucleic acid	Primer	Probe
Based on primer NSR1787/18 (ribosomal database project)	GGTTCACCTACRG	CYGCAGGTTCAC
Based on primer NSR1642/16 (ribosomal database project)	GGCGGTGTGTRC	GACGGGCG
Based on primer NSR1147/20 (ribosomal database project)	CAATTYYTTTRAGTTT	CCGTCAATTYY
Based on primer NSR1438/20 (ribosomal database project)	ATCACAGACCTGTTAT	GGGCATCACA
Based on primer NSR980/18 (ribosomal database project)	CGTTCTTGATYAA	ACTTTCGTTCTTGA
Based on primer NSR951/17 (ribosomal database project)	GYRAATGCTTTCGC	TTGGYRAATGC
Based on primer NSR581/18 (ribosomal database project)	CCGCGGCTGCTGGC	ATTACCGCG
Based on primer NSR399/19 (ribosomal database project)	GGCTCCYTCTCCGG	TCTCAGGCTCCY
Based on primers NSR399/19 and NSR387/18 (ribosomal database project)	CYTCTCCGGRRTCGARCCCT	TCTCAGGCTCCYTCTCCG

Specific mRNA/cDNA		
Target nucleic acid	Primer	Probe
<i>Yersinia enterocolitica</i> yadA gene	TTCTTTCTTTAATTGCGCGAC A	TTTTTCAATTTCTTTCT
<i>Yersinia enterocolitica</i> , vir F gene	ACTCATCTTACCATTAAAGAAG	AATATCAACAATACTCATCTT AC
<i>Yersenia enterocolitica</i> ail gene	CCAGTAATCCATAAAGG	GCAGCMCCCAGTAATC
<i>Staphylococcus aureus</i> nuc gene	TAGCCAAGCCTTGACGAACT	TAAGCAACTTTAGCCAAGCC
<i>Salmonella</i> sp. InvA	RCTGGTACTGATCGATAAT	AGACGRCTGGTACT
<i>B. anthracis</i> chromosomal gene rpoB	AACGATAGCTCCTACATTTGG AG	GGGGGACAAACGATAGCTC
<i>Listeria monocytogenes</i> mpl-gene	GCAACTTCCGGCTCAGC	AATCCAACCTCGCAACTTCCG
<i>Listeria monocytogenes</i> mpl-gene	GCATATCCCAWAGTTT	TATAAGCATATCCCA
<i>Listeria monocytogenes</i> Hly-a 1	TAGTTCTACATCACCTGAGAC AGA	TGATATTTGTTAGTTCTACA
<i>Listeria monocytogenes</i> Hly-a 2	TTCCGAATTCGCTTTTACGAG AGC	TTTTCTACTAATTCCGAA
<i>Listeria monocytogenes</i> Hly-a 3	CTTCTTCTTGCATTTTCCCTTC	AAACTAATGACTTCTTCTTG
Shigatoxin mRNA	CACAGACTGCGTCAGTGAGG	CGCTCTTGCCACAGACTG
	CGCTGCAGCTGTATTACTTTC	GAAACGCTGC
	TAAACTGCACTTCAGCAAAT	AGTCATTATTAACTGCA
Beta-lactamase mRNA 1:	TGCTTAATCAGTGAGGCACC	TTACCAATGCTTAATCAG
Beta-lactamase mRNA 2	RTAAATGCTTCAATAA	AACCCTGRTAAATGC

Universal bakterial 23S		
Target nucleic acid	Primer	Probe
Based on primer BLR1686/20 (ribosomal database project)	CATTTTGCCGAGTTC	GGGGCCATTTTGCCG
Based on primer	GACCGTTATAGTTAC	CTTAGGACCGT

BLR1929/20 (ribosomal database project)		
Based on primer BLR2595/20 (ribosomal database project)	TTCTGAACCCAGCTC	CGACGTTCTGAA
Based on primer BLR463/20 (ribosomal database project)	CTGGTTCACATATCGG	CGGTACTGGTT
Based on primer BLR2763/20 (ribosomal database project)	CTTAGATGCTTTCAGC	CCCGCTTAGATG

Universal eukaryote nuclear 28S

Target nucleic acid	Primer	Probe
Based on primer NLR677/16 (ribosomal database project)	GTCCACCAAGAT	ACTCGTCCACC
Based on primer NLR816/21 (ribosomal database project)	TCCGTGTTTCAAGAC	CCTTGGTCCGTGT
Based on primer NLR818/25 (ribosomal database project)	TCCGTGTTTCAAGACGG	CTCCTTGGTCCGTGT
Based on primer NLR1126/22 (ribosomal database project)	CTGAGGGAACTTCGG	GCTATCCTGAGG
Based on primer NLR1432/23 (ribosomal database project)	CACACTCCTTAGCGGA	GTTGTTACACACTCC
Based on primer NLR1694/17 (ribosomal database project)	GGAYCGACTNAC	TCTYAGGAYCG
Based on primer NLR2016/18 (ribosomal database project)	AGACCTGMTGCGG	CTTGGAGACCTG
Based on primer NLR2098/24 (ribosomal database project)	ATCCTTWTCCCGAAGTTAC	AGCCAATCCTTWTCC

Based on primer NLR2362/20 (ribosomal database project)	AGAGCACTGGGCAG	ACATTCAGAGCA
Based on primer NLR2368/26 (ribosomal database project)	AGAGCACTGGGCAGAAATCA	ACATTCAGAGCAC
Based on primer NLR2571/21 (ribosomal database project)	CAGGGTCTTCTTTCC	CTCAACAGGGT
Based on primer NLR2781/19 (ribosomal database project)	CCAGYCAAACCTCCC	CCGCCCCAGYC
Based on primer NLR3033/24 (ribosomal database project)	ACATCGAAGRATCAAAAAGC	GCCGACATCG
Based on primer NLR3113/24 (ribosomal database project)	CCAGCTCACGTTCCCT	GTCTAAACCCAGCTC
Based on primer NLR3284/21 (ribosomal database project)	TTAGAGGCGTTCAG	TTCTGACTTAGAGG

Virus detection (strand specific detection)		
Target nucleic acid	Primer	Probe
HIV RNA detektion (or + strand DNA detection)	TGCTATGTCACTTCCCCTTGG TTCTCT	TAGTAGTTCCTGCTATGTCA
HIV RNA detektion (or + strand DNA detection)	TACTAGTAGTTCCTGCTATGT CACTTCC	TCCTGAAGGGTACTAG
Hepatitis B RNA (or + strand DNA detection)	CGAACCACTGAACAAATGGC	AGCCCTACGAACCACTG
Hepatitis B - strand DNA detection	TCACCATATTCTTGGGAACAA GA	GCGGGTCACCATATT
Hepatitis C RNA + strand detection	CTCGCAAGCACCCATCAGG CAGT	CGGGGCACTCGCAAGCA
Hepatitis C RNA - strand detection	GCAGAAAGCGTCTAGCCATG GCGT	CTGTCTTCACGCAGAA
Hepatitis A RNA - strand	TTCTGCAGATTGGCTTACTAC	TTCTTGATTCACTCTGCA

detection		
Hepatitis A RNA + strand detection	CATGCAACTCCAAATCTGT	GAGTTAATCCATGCAAC
Rotavirus RNA + strand detection	CGAACAATTCTAATCTAAGAT	GGTCACATCGAACAATTC
Rotavirus - strand detection	ATGTACCGTGAAAGTGTGTCC GC	GATCATCATGTACCGTG
Zaire Ebola virus RNA - strand	ATGTGGTGGATTATA	ATTATAATAATCACTGACATG CAT
Zaire Ebola virus RNA + strand	ATCGGAACTTTTCTTTCT	TTCTTTCTCATTGAAAGA
SARS virus RNA + strand	AGAGCCTTGTCTTGG	GTTCTTGGTGTCAACGAGAA AACA
SARS virus RNA - strand	CACGAGTGAGTTCA	GAGTTCACGGAGTGCAC

In table 1, Y = C or T, S = C or G, M = C or A, R = A or G, W = A or T, K = G or T.

- As is the case for the target nucleic acid sequence, the nucleic acid molecule comprising a
5 sequence corresponding to the target nucleic acid sequence may be any type of nucleic
acid molecule. In a preferred embodiment of the invention, however, the nucleic acid
molecule comprising a sequence corresponding to the target nucleic acid sequence is a
genomic DNA molecule c.f. the classification of genomic DNA presented above.
- 10 When binding to a nucleic acid molecule which in part corresponds to the target nucleic
acid sequence the probe preferably prevents or reduces annealing of the forward
extendable primer. The probe may, however, also be designed so as to prevent or reduce
annealing of the reverse primer thereby abolishing the synthesis of a nucleic acid
molecule, which is complementary in sequence to the target nucleic acid molecule. In this
15 case the nucleic acid molecule comprising a sequence corresponding to the target nucleic
acid sequence is a nucleic acid molecule comprising a sequence which is complementary to
the target nucleic acid sequence.

- As indicated above the method according to the present invention may be applied to
20 conventional PCR reactions. A prerequisite for obtaining non-linear amplification of the
target sequence is the use of a forward primer together with a reverse primer. Therefore
the method may further comprise a step of contacting the sample with a second
extendable primer which is complementary to a part of the complementary strand of said
target nucleic acid sequence and detecting the target nucleic acid sequence subsequent to

its amplification. In the present context, the forward primer thus serves as a primer for the synthesis of a nucleic acid sequence, which is complementary to target nucleic acid or a part hereof whereas the reverse primer is a primer for the synthesis of a nucleic acid molecule, which is identical to the target nucleic acid or a part hereof.

5

In a preferred embodiment of the invention the T_m -value for the expected hybrid of the second extendable primer and the complementary strand of said target nucleotide sequence is comparable to the T_m -value for the expected hybrid of the nucleic acid probe and the nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence. This will make annealing of both the probe and the second extendable primer possible during the pre-primer annealing step serving the purpose of preventing re-annealing of the PCR products during the pre-primer annealing step. Furthermore it assures that replication of the nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence will proceed past the sequence corresponding to the target nucleic acid and into an adjacent part of the molecule.

Amplification of the target nucleic acid typically occurs through cycles comprising the steps of increasing or lowering the temperature of the sample to levels which will allow denaturation of nucleic acid hybrids and double stranded nucleic acids, annealing of the primers and probes, and replication of the relevant nucleic acids. In the method according to the invention the target nucleotide sequence and its complementary strand are thus amplified in at least one thermocycle comprising the steps of primer annealing, primer extension and denaturation.

It is to be understood that the temperature at which each step of the thermocycle is conducted may have to be optimised depending on the amplification method, the nature of the target nucleic acids, the extendable primers and the nucleic acid probe. Typically, however, the temperature of the denaturation step, the pre-primer annealing step, the annealing step and the elongation step is within the range of 90 - 99°C, 50 - 70°C, 40 - 65°C and 70 - 75°C, respectively. The duration of each step is from 5 seconds to 15 minutes, from 5 seconds to 5 minutes, from 10 seconds to 10 minutes and from 15 seconds to 15 minutes, respectively.

Typically, blocking probes are added to a final concentration within the range of 0.1 to 20 μ M whereas the primers are added to a final concentration within the range of 0.1 to 2 μ M.

While the Polymerase Chain Reaction is the most widely applied method for *in vitro* enzymatic amplification of nucleic acids, it is not unique. Several other techniques have been developed for particular applications. Ligase Chain Reaction, for instance, may be

used for the sensitive detection of a DNA sequence and for discrimination between alleles. Two primers are used which anneal at adjacent sites on target DNA. If the target sequence is present, the primers can be ligated together and will act as sites for further annealing and ligation. The strand displacement reaction is similar to the PCR reaction but does not
5 require a denaturation step in each cycle. After extension, the primers are separated from their extension product by restriction endonuclease cleavage and further extension then proceeds by strand displacement from the resulting nick. Transcription-based amplification or nucleic acid sequence-based amplification (NASBA) is a rapid amplification procedure based on transcription and reverse transcription. The target nucleic acid is amplified by
10 standard techniques using primers carrying a phage promoter and further amplification is then carried out by transcription using phage RNA polymerase. The transcripts are reverse transcribed to generate cDNA for further amplification. The amplification of nucleic acids which is associated with the method of the present invention may occur by any *in vitro* amplification method, in particular in a PCR or a LCR procedure.

15 Numerous advances and extensions to basic PCR strategy have been developed. The PCR amplification of cDNA is termed RT-PCR as it involves the initial reverse transcription of RNA prior to amplification. Several applications of the RT-PCR technique have been devised such as the identification of differentially expressed genes known as differential display
20 PCR or mRNA fingerprinting. Another PCR based approach is asymmetric PCR wherein one of the primers becomes depleted after a limited number of cycles. It is to be understood that the method of the present invention may be applied to virtually any PCR based technique including those mentioned. In a preferred embodiment of the invention, however, the amplification of a target nucleic acid sequence takes place as an integrated
25 part of a RT-PCR procedure. When applying the method of the present invention to a RT-PCR procedure, the main objective may be to prevent amplification of genomic DNA that is identical or partly identical to the complementary DNA formed. Alternatively, the method may be applied in order to prevent or reduce the amplification of contaminating nucleic acid species, which unintentionally have been introduced into a sample during preparation
30 or handling procedures.

In a further preferred embodiment of the invention the sample is contacted with the nucleic acid probe prior to reverse transcription of the RNA. In this case, which may be referred to as a so-called 'one-step' RT-PCR procedure, the nucleic acid probe will not be
35 able to interfere with annealing of the extendable primers to the RNA sequence, which is subject to reverse transcription and subsequent amplification and detection. In the present context, the term 'one-step' indicates that generation of cDNA from the RNA and amplification of the cDNA takes place in successive steps of a single procedure during which there may be no need to add further reagents. The possibility of conducting the

method of the present invention as a one-step RT-PCR procedure exists when the nucleic acid probe is designed to anneal to a sequence of the genomic DNA comprising the transcription initiation site of a gene and the first extendable primer has a sequence within the cDNA corresponding to the transcription initiation sequence as its target.

5

Optionally, detection of the presence of the target nucleic acid is performed using a real-time PCR system. All real-time PCR systems rely upon the detection and quantification of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. In a simple and economical format, that reporter is the double-strand DNA-specific dye SYBR® Green (Molecular Probes). SYBR Green binds double-stranded DNA, and upon excitation emits light. Thus, as a PCR product accumulates, fluorescence increases. The two most popular alternatives to SYBR Green are TaqMan® and molecular beacons, both of which are hybridisation probes relying on fluorescence resonance energy transfer (FRET) for quantification. TaqMan Probes are oligonucleotides that contain a fluorescent dye, typically on the 5' base, and a quenching dye, typically located on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. TaqMan probes are designed to hybridise to an internal region of a PCR product. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. This separates the fluorescent and quenching dyes and FRET no longer occurs. Fluorescence increases in each cycle, proportional to the rate of probe cleavage. Molecular beacons also contain fluorescent and quenching dyes, but FRET only occurs when the quenching dye is directly adjacent to the fluorescent dye. Molecular beacons are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye and quencher in close proximity. When a molecular beacon hybridises to a target, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation.

30 By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during the 3-15 cycles (by default) indicates the detection of accumulated PCR product. A fixed fluorescence threshold is set significantly above the baseline, which can be altered by the operator. The parameter C_T (threshold cycle) is defined as the cycle number at which the fluorescence emission exceeds the fixed threshold.

One additional possibility, which may be particularly feasible considering the advance of the real-time PCR technology, is thus to detect the presence of the target nucleic acid molecule in a quantitative or semi-quantitative manner. Typically, this is accomplished by determining the amount of product generated by amplification of the target nucleic acid
5 sequence, or in real-time PCR the corresponding C_T -value, relative to the amount of product or the C_T -value obtained when amplifying a nucleic acid sequence, which is present in the sample in a known quantity. Thus, the method of the present invention may further comprise steps of determining the quantity of a target nucleotide sequence in a plurality of nucleic acid molecules.

10

The method of the present invention may advantageously be applied when seeking to detect the presence of microbial species in general and also when aiming at identifying or characterising a microbial species present. Moreover the technique is applicable when seeking to characterise nucleic acid sequences present within a known microbial species,
15 or nucleic acids which are synthesised in response to the presence of a microbial species. It may therefore for certain applications be preferred that the first extendable primer is complementary to a nucleotide sequence, which is specific for one or more microbial species. By a microbial species is meant a micro-organism such as a bacterium, archaean, protistan, virus as well as fungi or related small eucaryot species.

20

Of particular relevance may also be the detection or determination of nucleic acid sequences conferring resistance towards antibiotics whether such resistance is desired or not. Accordingly, the first extendable primer may be complementary to a nucleotide sequence of a gene the product of which confers resistance towards one or more
25 antibiotics.

An objective of employing the method according to the present invention may for instance be to detect the presence of microbial infections possibly causing a poisoning reaction. Therefore, it may be preferred that the first extendable primer is complementary to a
30 nucleotide sequence of a gene encoding a microbial toxin. A large array of microbial toxins is known including among others the bacterial toxins. The latter include the exotoxins which are conventionally classified into three types: enterotoxins, cytotoxins, and neurotoxins, as well as the endotoxins. A few examples of bacterial toxins are botulinum toxin, tetanus toxin, diphtheria toxin, streptolysosins O and S, Cholera toxin, heat-labile
35 enterotoxin, heat-stable enterotoxin, and shiga toxin.

The method of the invention may be employed in the detection of nucleic acid molecules in samples, which are obtained from a multicellular organism and may be used as part of a diagnostic procedure. In particular, the sample may contain body fluids such as blood,

serum, urine or saliva as well as body tissue, for example skin, muscle or bone, or it may contain extracts or homogenates of such body tissue. Preferably, the sample is obtained from a mammal, such as a primate. In particular, the sample may be obtained from a human.

5

Alternatively the method of the invention may be applied to samples, which are obtained from a food source or from a source of drinking water and the objective may be to evaluate these sources of food and/or drinking water. Of particular relevance would be the detection of contaminants within such environments, which may constitute a health issue,

10 such as the detection of pathogenic organisms.

As for application of the method according to the invention to the detection of *Salmonella* Spp, salmonella live in the intestinal tracts of humans and other animals including birds, and are transmitted by the oral-fecal route, that is via intake of foods contaminated with
15 animal feces. Contaminated foods are often of animal origin, such as beef, poultry, milk, or eggs, but all foods, including vegetables may become contaminated. For detection of salmonella, samples may thus be obtained from virtually any food sources, in particular any of those mentioned above.

20 As mentioned above the microbial species may be a bacterial species. The bacterial species may belong to any of the constituent groups of the archaeobacteria or eubacteria. Constituent groups of archaeobacteria are the *Sulfolobus-Thermoproteus* group, the methanogen-halophile group and the *Thermoplasma*. Constituent groups of eubacteria may be the gram-positive eubacteria, the purple bacteria - pseudomonad group,
25 spirochetes, the bacterioides-cytophaga group, the cyanobacteria, the green sulphur bacteria, the green non-sulphur bacteria, the sulphur-reducers and muxobacteria, the radioresistant micrococci and the planctomyces group. More specifically, the bacterial species may be selected from the group comprising: *Salmonella* spp., *Escherichia* spp., *Listeria* spp., *Shigella* spp., *Legionella* spp., *Campylobacter* spp., *Staphylococcus* spp.,
30 *Clostridium* spp., *Streptococcus* spp., *Vibrio* spp., *Bacillus* spp., *Yersenia* spp., *Mycobacterium* spp.

Other than being a bacterial species the microbial species may also be an animal, plant or bacterial virus and may belong to any family within these classes. Furthermore the virus
35 may be a DNA virus, such as a dsDNA virus or a ssDNA virus, as well as a RNA replicating virus, such as a dsRNA virus, a (-)ssRNA virus or a (+)ssRNA virus. Alternatively, the virus may be a RNA- or DNA-retroid virus. The method of the invention may be conveniently used in strand-specific RT-PCR procedures for instance for detection of retroviral RNA and/or detection of one of two complementary strands of a viral genome. In particular, a

strand specific RT-PCR approach is useful when seeking to detect viruses, which utilise the synthesis of replicative RNA, a negative-strand RNA complementary to the genomic nucleic acid, as a template for virus replication.

- 5 Although the invention is exemplified in the present text by the use of only one nucleic acid probe it is to be understood that, at least in theory, any number of different probes may be used in the method of the present invention. In a PCR reaction, for instance, it may be feasible to use one probe which prevent annealing of a forward primer to a nucleic acid molecule corresponding to the target nucleic acid sequence in combination with
10 another probe that prevents annealing of a reverse primer to a nucleic acid molecule corresponding to a sequence which is complementary to the target nucleic acid sequence. Thus, the method according to the invention may further comprise the step of contacting the sample with at least one second nucleic acid probe capable of binding to a continuum of at least a part of the nucleic acid molecule corresponding to a sequence complementary
15 to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence.

- As described above for the nucleic acid probe the at least one second nucleic acid probe may be composed of two parts. Therefore, in a preferred embodiment of the invention the
20 at least one second nucleic acid probe comprises a first part, which is complementary to a 3' region within said part of the nucleic acid molecule corresponding to a sequence complementary to the target nucleic acid sequence and a second part, which is complementary to a neighbouring sequence of the nucleic acid molecule.

- 25 The use of more than one nucleic acid probe may also be instituted when more than one molecular species, which are structurally related to a target molecule are present especially in cases where the use of only one probe is insufficient. Finally, one can also imagine that several different target nucleic acids are to be detected in a multiplex reaction, one or more sets of primers and probes being used for each target nucleic acid.

30

- A second aspect of the invention relates to a composition for use, or a composition useful in the detection of the presence or absence of at least one target nucleic acid sequence in a sample that contains a nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence, said composition comprising at least one nucleic acid probe
35 that is capable of selectively binding to a continuum of at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence.

In addition to the nucleic acid probe, a composition according to the invention may comprise any number of additional components. In particular the composition may comprise at least one member selected from the group consisting of polymerases, nucleotides, buffers, salts, stabilising agents, diluents and agents for detection of nucleic acids. These may be reagents, which recognise specific sequences or reagents, which recognise nucleic acids in a non-specific manner. Examples of the former are oligonucleotides or nucleic acid analogues optionally bearing detectable labels. Examples of the latter are antibodies to nucleic acids.

10 In another aspect of the invention, a kit for detecting the presence or absence of one or more target nucleic acid sequence in a sample that contains one or more related nucleic acid molecules. Again, a related nucleic acid molecule may be a nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence. The kit may contain one or more of the reagents necessary for detecting, hereunder identifying, the particular target nucleic acid or acids including primers, non-extendable blocking probes, reaction buffers, as well as directions giving a protocol for carrying out the procedure. In particular, the kit may comprise at least one nucleic acid probe that is capable of selectively binding to a continuum of at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence.

The kit may further comprise at least one member selected from the group consisting of polymerases, nucleotides, buffers, salts, stabilising agents, diluents and agents for detection of nucleic acids. Again, these may be reagents, which recognise specific sequences or reagents, which recognise nucleic acids in a non-specific manner. Examples of the former are oligonucleotides or nucleic acid analogues optionally bearing detectable labels. Examples of the latter are antibodies to nucleic acids.

It is to be understood that any part of the description relating to the first aspect of the invention, namely the method itself, will also apply to the composition and the kit of parts described above.

FIGURE LEGENDS

35

Figure 1.

Inhibitor probe principle: Following annealing of the blocking probe to a nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence (DNA) attachment of the primer to said molecule and/or extension of said primer is blocked under

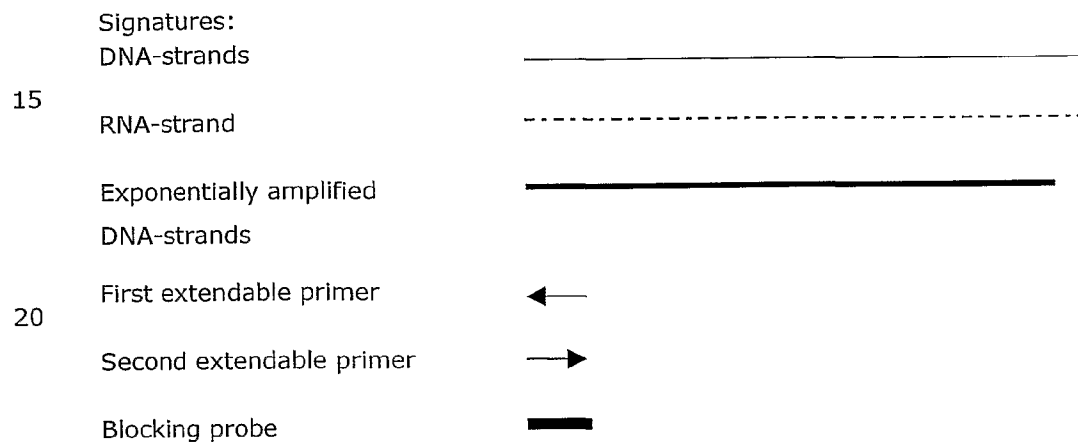
conditions where attachment and/or extension of said primer to the target nucleic acid sequence (cDNA) is possible. Thereby amplification of the target nucleic acid is allowed while amplification of sequence corresponding to the target nucleic acid sequence is blocked.

5

Figure 2.

RT - PCR using blocking probes: In the RT-reaction reverse transcription of an RNA template is performed using a first extendable primer is. In the subsequent PCR reactions the blocking probe will block the DNA strand containing the sequence corresponding to the target sequence and the amplification products of the complementary DNA strand. Thus,

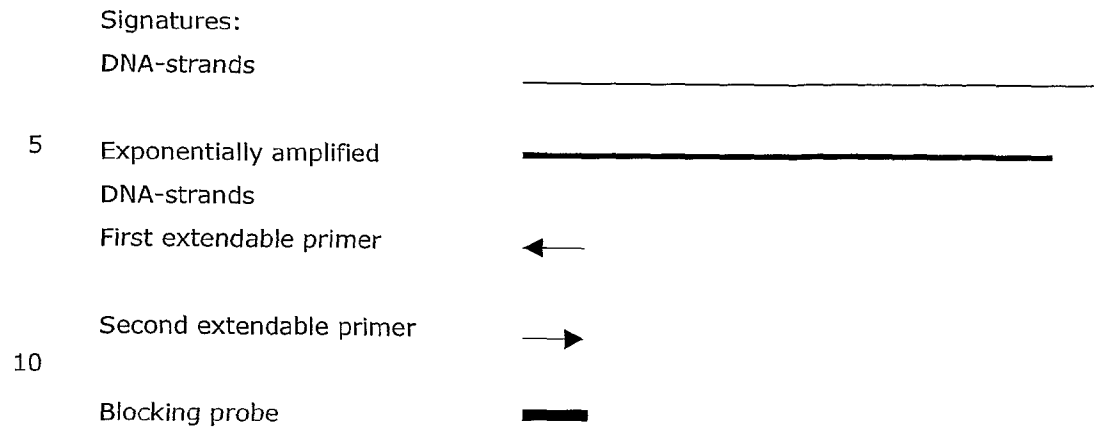
10 the PCR reaction results in exponential amplification of only the target sequence.



Figur 3.

Strand specific PCR using blocking probes: In the first PCR reaction amplification of the target strand is done using a first extendable primer. In the following PCR reactions the blocking probe will block the DNA strand containing the sequence complementary to the target sequence and the amplification products of this complementary DNA strand. Thus,

30 the PCR reaction results in exponential amplification of only the target strand.

**Figure 4.**

15 Ethidium bromide stained PCR-products using mixtures of genomic *gfp* containing DNA derived from *P. putida* OUS82 UCB55 and *gfp* internal standard as template. Conc. of internal standard was kept constant in all tubes while the conc. of genomic *gfp* was consistently halved in tubes 1-8. **Upper panel:** No blocking probe added. Approx. equal band intensities measured in lane 7 implying equal amount of templates initially present in

20 tube 7. **Middle panel:** PNAB1 added (8,0 μ M final conc.) to PCR reactions. Approx. equal band intensities in lane 1 implying approx. equal amplification in the initial presence of more than 2^6 times more *gfp* template than internal standard template. **Lower panel:** DNAB1 added (8,0 μ M final conc.) to PCR reactions. Approx. equal intensities in lane 4 implying some suppression of the *gfp* template.

25

Figure 5.

Ethidium bromide stained RT-PCR product bands using DNase treated (lanes 1-4) and untreated (lanes 5-8) total RNA extracts from *P. putida* OUS82 UCB55. RT enzyme was excluded from reactions 3, 4, 7 and 8 (products shown in lanes 3, 4, 7, and 8 respectively)

30 and PNAB1 (8.0 μ M final conc.) was added to PCR-reactions 2, 4, 6 and 8 (products shown in lanes 2, 4, 6 and 8, respectively).

Figure 6.

Ethidium bromide stained RT-PCR product bands using as template DNase treated (lanes 6-10, 16-20), and untreated (lanes 1-5, and 11-15) total RNA extracts from 5 parallel cultures of *E. coli* SP308/pKJK10. Increasing levels of IPTG were used to induce each culture. PNAB1 was added (8.0 μ M final conc.) to reactions 11-20 (lanes 11-20, respectively).

Figure 7.

Ethidium bromide stained RT-PCR product bands using as template DNase treated (lanes 1-4 a, b), and untreated (lanes 5-8 a, b) total RNA extracts from exponentially growing (a) and starving (b) cultures of *E. coli* SP308/pKJK10. RT enzyme was excluded from reactions 3, 4, 7 and 8 (products shown by lanes 3, 4, 7, and 8 a, b respectively) and addition of PNAB2-reverse (2.0 μ M final conc.) to PCR-reactions 2, 4, 6 and 8 was performed (products shown by lanes 2, 4, 6 and 8, a, b respectively).

Figure 8.

Agarose gel with bands representing products originating from Real-time PCR analysis presented in Tabel 3. Lane 1: Direct nucleic acid extract (RNA and DNA), with no reverse transcriptase treatment but with addition of PNAInvA-reverse 1. Lane 2: like lane 1 but no PNA added (*corresponding to table 3, 2. row, No PNA*); Lane 3: Reverse transcribed after DNA'se treatment with PNA and lane 4: like 3 but with no PNA addition (*corresponding to table 3, 3. row, with PNA and no PNA, respectively*); Lane 5: Reverse transcribed, no DNA'se treatment, PCR with PNA probe and lane 6 like Lane 5 but without PNA addition (*corresponding to table 3, 4. row, with PNA and no PNA, respectively*), Lane 7 Molecular ladder; Lane 8: negative control.

EXAMPLES**Materials and experimental conditions****Strains:**

Two bacterial strains, *Escherichia coli* SP308/pKJK10 (16) and *Pseudomonas putida* OUS82 UCB55 (16), harbouring the same *gfp* construct (17) were used in this study. The *gfp* gene in *E. coli* SP308/pKJK10 was under the control of the LacI repressible promoter $P_{A1/04/03}$ (18) and is inducible by the gratuitous inducer of the lac operon isopropyl- β -D-thiogalactopyranoside (IPTG). In the *P. putida* strain the *gfp*-gene was constitutively transcribed. The *S. typhimorium* invA gene was constitutively transcribed.

DNA and RNA extractions:

Pure cultures of *P. putida* OUS82 wt (16, 17) and *P. putida* OUS82 UCB55 were grown overnight in 10 % LB broth and DNA was extracted using the Fast Soil DNA purification kit (Bio 101, Vista, Calif.) modified for liquid samples.

Total RNA was extracted using the FASTRNA blue kit (Bio 101) in accordance with the manufacturers instructions. *P. putida* OUS82 UCB55 was grown to exponential phase in 10% LB broth. Approx. 10^7 cells (200 μ l, OD₆₀₀ = 0.1) were sampled and total RNA was
5 extracted.

Five parallel cultures of *E. coli* SP308 cells were grown exponentially in minimal glucose media supplemented with casaminoacids (0.5 % final conc.). DNA was extracted using the Fast Soil DNA purification kit (BIO101). At time 0, the five cultures received IPTG to final
10 concentrations of 0 μ M, 100 μ M, 500 μ M, 1000 μ M or 2000 μ M, respectively. Cell numbers and fluorescence per cell was monitored every 30 min. by flow cytometry (BD FACSCalibur, FSC= E01, SSC= 350, FL1= 566 (λ =530 \pm 15 nm). All on log gain). After 2 hours of exponential growth in the presence or absence of IPTG, duplicates of 10^7 cells were sampled and immediately frozen in liquid nitrogen. Samples were kept at -80°C until total RNA was
15 extracted.

Total RNA was further extracted from 10^7 starved *E. coli* SP308 cells grown to lag phase in minimal glucose media supplemented with casaminoacids and thereafter incubated for 3
20 days.

Pure culture of *S. typhimurium* was grown in full strength LB over night and the DNA was extracted using the MO BIO ultraclean soil DNA extraction kit (MO-BIO, Solane Beach, CA). All DNA extractions were done as recommended by the manufacturer except replacing the soil with pure culture. For RNA isolation *S. typhimurium* was grown overnight at RT in full
25 strength LB medium and 300 μ l culture was washed and resuspended in 300 μ l phosphate buffer pH 7.4. RNA was extracted from the suspended cells using the BIO101 Fast RNA kit for soil in accordance with the manufacturers instruction.

All RNA samples were extracted to a final volume of 100 μ l. All RNA samples were reverse
30 transcribed immediately after extraction or treated with DNase and thereafter reverse transcribed. All reagents used in the procedures was either treated and labelled RNase free by supplier or treated with 0.1% DEPC Diethyl pyrocarbonate (Sigma, St. Louis, MO, USA) prior to autoclaving to produce RNase free reagents.

35 DNase treatment of RNA samples from *P. putida* and *E. coli* was performed using the Amplification Grade DNase I kit (SIGMA, St. Lou., Miss.) in accordance with the manufacturers instructions. DNase treatment of samples from *S. typhimurium* was performed using RQ1 Rnase-free DNase (Promega, Madison, WI, USA).

Primers and blocking probes:

Primers and blocking probes used in this study are shown in table 2. *gfpmb1-Fx* was designed to target the leading strand sequence corresponding to the expected start sequence of the reverse transcribed *gfp* messenger -10 bases from the pribnow box. *gfpmb2-R* was placed approximately 770 bases downstream of *gfpmb1-Fx*. To prevent re-annealing of PCR products during the pre-primer-annealing step and to assure that replication of the genomic DNA lagging strand proceeds through the transcription initiation point, the reverse primer was designed to anneal in the pre-primer-annealing step of the PCR reaction.

Table 2:

Primer/Probe	Sequence
<i>gfpmb1-Fx</i>	5'AATTGTGAGCGGATAACA3'
<i>gfpmb2-R</i>	5'AAGCTTATTTGTATAGTTCATCCAT GC 3'
PRBA338F	5'ACTCCTACGGGAGGCAGCAG 3'
511R2	5'GCTGGCACGGAG 3'
InvAF2	5'ACA GTG CTC GTT TAC GAC-3'
InvAR1	5'ACT GGT ACT GAT CGA TAA-3'
DNAB1	5'GATAACAATGATACTAGATTCAATT GTGAGCGGA 3'Biotin
PNAB1	H-ACTAGATTCTGATTGTG AG --NH ₂
PNAB2- reverse	H-GCGGCTGCTGG-NH ₂
PNAInvA- reverse-1	H-AGA-CGA/C-CTG-GTA-CT-NH ₂

Two different blocking probes DNAB1 and PNAB1 were designed targeting the leading strand region immediately upstream of and just overlapping the *gfpmb1-Fx* target. To avoid secondary structures detrimental to synthesis of the PNA probe a mismatch was introduced

in the PNA probe. The mismatch was introduced to the region of the probe overlapping the *gfp* cDNA sequence. By this placement of the mismatch, the expected T_m value of the PNA/cDNA hybrid (1 mismatch/10match) was lowered while the expected T_m of the PNA/genomicDNA hybrid was not affected to the same extent (1 mismatch/18match). An A to G change was chosen as such a substitution was expected to have the least impact on T_m (10).

The primers PRBA338F and 511R2 were used to amplify rRNA from *E. coli*. PNAB2-reverse was designed to block amplification of rDNA from the reverse primer. This approach limits the use of the system to a two step RT-PCR procedure, as a probe present in the RT step would inhibit cDNA synthesis. However the general applicability of the developed technique is greatly enhanced as it liberates the researcher from design restrictions arising due to the transcription initiation placement of the primer/probe pair when blocking the forward primer.

The primers invAF2 and invAR1a/invAR1b were designed to target the *invA* gene from *Salmonella* sp. involved in the invasiveness of *Salmonella*; this gene appears to be a valid target for most *Salmonella* (Chen and Griffith 2001; Malorney *et al.* 2003)

Primers and DNAB1 were custom primers of HPSF purity purchased from MWG Biotech AG, Germany. PNAB1 and PNAB2-reverse and PNAinvA-reverse-1 were purchased from Applied Biosystems.

PCR conditions:

Gfp-targeted PCR was performed in a final volume of 25 μ l, containing 17.5 μ l or, when blocking probes were added, 15.5 μ l DNase/RNase-free water (Bio 101, Vista, Calif.), 2.5 μ l 10X Ampli *Taq* PCR-buffer (Applied Biosystems, Norwalk, Conn.), 0.125 μ l of each primer (in a 0.1 mM solution), 2.5 μ l of DNase-free bovine serum albumin (Amersham Pharmacia, Uppsala, Sweden), 1 μ l of Gene Amp 10 mM deoxynucleoside triphosphate mixture (Applied Biosystems), 0.125 μ l of Ampli *Taq* polymerase (Applied Biosystems) and 1 μ l of template DNA. Blocking probe was added to a final concentration of 8.0 μ M (2 μ l of a 0.1 mM solution). Cycling conditions; 10 min at 95°C; 35 cycles of 30s at 95°C, 30s at 63°C, 30s at 57°C and 1 min at 72°C; 6 min at 72°C and final cooling at 4°C. rRNA-targeted PCR cycling conditions; 10 min at 95°C; 35 cycles of 30s at 92°C, 30s at 58°C, 30s at 53°C and 1 min at 72°C; 6 min at 72°C and final cooling at 4°C.

PCR of cDNA was done as described above except for that 3 μ l of the obtained cDNA product was used in a 50 μ l reaction containing; 29.7 μ l or, when blocking probe was

added, 25.7 µl RNase-free water (Bio 101), 9.4 µl 5X RT-PCR buffer, 3.2 µl 25 mM magnesium chloride, 3.7 µl 10 mM dNTP blend, 0.25 µl of the primers (in a 0.1 mM solution), 0.5 µl Ampli Taq Gold DNA polymerase (2.5 units) and when appropriate 4.0 µl PNAGFP1 (in a 0.1 mM solution to achieve 8.0 µM final conc.).

5 PCR cycling conditions as above.

Gfp-targeted RT-PCR was performed as a two step reaction using the GeneAmp Gold RNA PCR reagent kit (Applied Biosystems) in accordance with the manufacturers instructions. 1 µl of template RNA was reverse transcribed in a 20 µl reaction volume (15 min. at 45°C)
 10 containing: 8.1 µl RNase-free water (Bio 101), 4.0 µl 5X RT-PCR buffer, 2.0 µl 25 mM magnesium chloride, 2.0 µl 10 mM dNTP blend, 0.5 µl RNase inhibitor (10 units), 2.0 µl 100 mM DTT, 0.1 µl *gfpmb2*-R primer and 0.3 µl MultiScribe reverse transcriptase (15 units). 3 µl of the obtained product was used in the subsequent PCR step containing; 29.7 µl or, when blocking probe was added, 25.7 µl RNase-free water (Bio 101), 9.4 µl 5X RT-PCR
 15 buffer, 3.2 µl 25 mM magnesium chloride, 3.7 µl 10 mM dNTP blend, 0.25 µl of the primers (in a 0.1 mM solution), 0.5 µl Ampli Taq Gold DNA polymerase (2.5 units) and when appropriate 4.0 µl PNAB1 (in a 0.1 mM solution to achieve 8.0 µM final conc.). PCR cycling conditions as above.

20 rRNA-targeted RT-PCR was performed as a two-step procedure using ImProm-II reverse transcriptase and *Taq* DNA polymerase (Promega, Madison, WI.) in accordance with the manufacturers instructions. 1 µl of heat-denatured (70°C for 5 min.) template RNA was reverse transcribed in a 20 µl reaction volume (30 min. at 45°C) containing: 10.4 µl RNase-free water (Promega), 4.0 µl 5X Improm-II reaction buffer, 2.4 µl 25 mM magnesium
 25 chloride, 1.0 µl 10 mM dNTP blend, 0.2 µl (20 pmol) 511R-2 primer and 1.0 µl Improm-II reverse transcriptase. 2 µl of the obtained product was used in the subsequent PCR step containing; 22.75 µl or, when blocking probe was added, 21.75 µl Nuclease-free water (Promega), 25 µl 2X PCR Master mix (Promega), 0.125 µl of the primers (in a 0.1 mM solution) and when appropriate 1.0 µl PNAB2-reverse (in a 0.1 mM solution to achieve 2.0
 30 µM final conc.). PCR cycling conditions as above.

For Real Time PCR of *S. thyphimorium* *InvA* cDNA PCR reactions in a total volume of 25 µl was prepared by combining: 13.25 µl dH₂O, 2.5 µl 10X reaction buffer (BIO RAD); 0.75 µl MgCl₂, 1 µl 5mM dNTP mixture, 0.5 µl 20 pmol/µl InvAF2 and InvAR1a/ InvAR1b primers;
 35 0.25 µl 1/1000 diluted cybergreen and 0.25 µl iTaq polymerase (BIO-RAD). PCR conditions in the BIORAD real time thermocycler: 10 min at 95°C; 35 cycles of 30s at 95°C, 30s at 67°C, 30s at 55°C and 30s min at 72°C; followed by a melting program with 0.5°C increase between each measurement using FAM490 filters, finally followed by cooling at 4°C

PNAB1 and PNAB2-reverse stock solutions were vortexed and heated to 50°C for 10 min. prior to addition to PCR mastermix. Estimates of PCR-product abundance were obtained by quantification of ethidium bromide-stained PCR product band intensities on 1.5% agarose gels using an UV-visible-light recording camera and the Multi-Analyst software (Bio-rad, Hercules, Calif.)

Example 1

PNAB1 inhibits the formation of genomic derived *gfp* PCR product.

10

PCR results when using template DNA from *P. putida* OUS82 wt and *P. putida* OUS82 UCB55 confirmed the *gfp* specificity of the primers (data not shown).

Formation of *gfp* PCR product using *P. putida* OUS82 UCB55 genomic DNA as template was monitored at annealing step temperatures between 55°-65°. Most product was observed at annealing temperatures 57° and 58°, while slightly less product was observed at 59°, and no product was observed at 60° (data not shown). The PCR cycling program (see PCR conditions) was decided on the basis of these results. The probes require the sequence upstream of the forward-primer target sequence in order to block amplification. Once a *gfp* PCR-product of genomic DNA origin is produced it is subsequently "immune" to the blocking probe and is amplified at the same exponential rate as true mRNA derived cDNAs. Although PCR on genomic DNA with or without blocking probes (especially using the PNA probe) generated substantially differing amounts of product a total block of genomic *gfp* was not observed (data not shown). In order to assess the extent of inhibition we used a DNA fragment (*gfp* internal standard) (21) differing in length by an internal deletion from the transcribed part of the *gfp* sequence (between primers *gfpmb1*-Fx and *gfpmb2*-R). The internal standard DNA fragment has the exact same 3'end and 5'end as the expected *gfp* cDNA, and a mix of *gfp* internal standard DNA fragment and genomic DNA containing the *gfp* locus was used to mimic samples of mixed *gfp* cDNA and the homolog genomic DNA fragment. This is reasonable as the internal standard and the *gfp* sequence have the same primer target sites and PCR can be carried out at approximately similar amplification rates. Differing in size, *gfp* and *gfp* internal standard are readily separated on a gel and the ratio between PCR products resemble the ratio initially present in the sample. PCR was run with or without blocking probes using mixtures of *gfp* internal standard and genomic DNA derived from *P. putida* OUS82 UCB55 as template. The concentration of internal standard template was kept constant in all reactions while the concentration of genomic DNA was consistently halved over a series of 8 reactions. Results are shown in figure 4.

DNAB1 did not block the genomically derived *gfp* satisfactory, only moving the product equilibrium three steps to the left (figure 4, upper vs. lower panel).

Raising the blocking probe concentration resulted in suppression of both *gfp* and *gfp*-
5 internal standard (data not shown). PNAB1, however, moved the equilibrium more than 6
steps to the left (figure 4, upper vs. middle panel). Raising the probe concentration did not
result in increased suppression of *gfp* PCR product (data not shown). Due to the higher
stability of a PNA/DNA duplex when compared to a DNA/DNA duplex, the event of primer
invasion and subsequent amplification seems more likely using a blocking probe consisting
10 of DNA than when using a PNA probe. The poor performance of the DNAB1 probe was
therefore not surprising. Though the PNAB1 did not block the amplification of genomic *gfp*
totally, it did suppress the amplification by approx. 2^6 times. Hence less than 2% of the
actual amount of contaminating genomic *gfp* was able to escape suppression and generate
a PCR-product. Additionally, using PNAB1 seemed to enhance the overall PCR-reaction.
15 Additions of non-specific oligonucleotides (tRNA) to (RT-) PCR reactions have been shown
to increase product formation, presumably by binding loosely to excess primers during the
initial amplification cycles (22). The enhanced PCR-performance after addition of PNAB1 is
probably caused by a similar effect.

20

Example 2

**PNAB1 blocks the formation of genomic *gfp* derived RT-PCR product, while
enhancing RT-PCR amplification of *gfp* mRNA using *P. putida* OUS82 UCB55 RNA
25 samples as template.**

Duplicate samples of total RNA were extracted from approx. 10^7 exponentially growing *P.*
putida OUS82 UCB55 cells constitutively transcribing the *gfp* gene. One sample was
reverse transcribed immediately and the other sample was treated with DNase prior to
30 reverse transcription. Reverse transcription was performed with and without the RT-
enzyme and the following PCR was performed with and without the PNA probe. Results are
shown in figure 5.

The presence of DNA in the RNA extractions was confirmed (lane 7) and DNase treatment
35 efficiently removed contaminating DNA (lane 3 and 4). A similar "removal" was observed
using the PNA blocking probe (lane 8). By comparing lanes 1, 2 and 6 it seems evident
that the PNA probe has a beneficial effect on the overall PCR performance (1 vs. 2) and
that the DNase treatment has a negative effect on the quantity of mRNA-derived PCR
product (2 vs. 6) (relative light intensities from products in lanes 1-8 respectively: 1.00;

1.42; 0.01; 0.06; 3.21; 2.72; 2.23; 0.15). These effects were confirmed when repeating the experiment and raising the number of PCR-cycles to 40. However when raising the number of PCR-cycles to 40 a visible band occurred in lane 8 (data not shown).

5

Example 3

Quantitative RT-PCR estimates of *gfp* mRNA correlate to Gfp protein quantitations when using PNAB1 to block genomic *gfp* DNA.

10

The PNA blocking probe was further tested using total RNA from the IPTG inducible strain *E. coli* SP308/pKJK10. Total RNA from 10^7 cells was extracted in duplicates from 5 parallel cultures of *E. coli* SP308/pKJK10, grown exponentially for 2 hours in the presence of final IPTG concentrations of 0 μ M, 100 μ M, 500 μ M, 1000 μ M and 2000 μ M, respectively. The cell
15 density did not vary between cultures whereas fluorescence above background levels was measured in the cultures treated with 500 μ M, 1000 μ M and 2000 μ M IPTG. Relative fluorescence intensities per cell at the time of sampling were 1.24; 1.29; 3.15; 7.95 and 16.30, respectively. Cell samples were immediately frozen in liquid nitrogen and kept at -80°C until total RNA was extracted. All RNA samples were reverse transcribed immediately
20 after extraction or treated with DNase and thereafter reverse transcribed. The following PCR was performed with or without blocking probe. Results are shown in figure 6.

Again the presence of DNA in the RNA extractions was confirmed and without DNase-treatment or blocking probe, no difference in the amount of RT-PCR product between different IPTG-treatments could be detected (lane 1-5) (relative light intensities were 1.00;
25 1.28; 0.71; 1.26; 0.96). DNase-treatment of samples efficiently removed DNA and in agreement with the FACS-data, RT-PCR product was detected in samples induced with 500, 1000 and 2000 μ M IPTG (lane 6-10). However, quantitative estimates obtained by measuring light intensities of the ethidium bromide stained bands (-0.01; 0.00; 0.32; 0.52; 0.22) did not show the expected differential induction levels from different IPTG
30 treatments. Adding PNAB1 to PCR reactions using the DNase treated samples as template, did again enhance the reaction generating increased amounts of product (lane 16-20)(relative light intensities of band were; -0.02; 0.00; 0.92; 0.60; 0.73).

When adding PNAB1 to the PCR step of the RT-PCR reaction using the non-DNase treated
35 samples as template product of the expected size was detected in all treatments (lane 11-15) (relative light intensities were 0.43; 0.26; 0.71; 2.41; 2.66). This suggest that either PNAB1 did not block the genomic DNA or *E. coli* SP308/pKJK10 has a leaky expression of the *gfp* gene. Though no RT-PCR product could be generated after DNase treatment, the latter explanation seems the more likely since *E. coli* SP308/pKJK10 carries the *gfp*-gene

on a multi-copy plasmid and the lac repressor is present as a single copy on the chromosome. That *E. coli* SP308/pKJK10 express *gfp* to some extent even in the absence of IPTG is also indicated by a lower average fluorescence intensity per cell of the parental strain without the *gfp*-containing plasmid (data not shown). A small amount of messenger
5 could, if present, have been degraded during the DNase-treatment, while giving rise to a RT-PCR product in the samples not treated with DNase. However, only small amounts of product were detected in the non-induced sample and product increased with the amount of IPTG added. An unexpected product of approx.1200 bp. was observed in all treatments. The presence of genomic DNA in the RT-PCR was apparently required for this product to
10 arise, since this product was not present in the DNase treated samples. In a high stringency Southern analyses, a probe consisting of radioactively labelled *gfp* PCR-product was observed to hybridise to both bands, thus indicating the presence of the *gfp* sequence in both PCR fragments (data not shown). Furthermore, the amount of the 1200 bp product seems to be inversely related to the ratio between *gfp* mRNA and genomic *gfp* DNA
15 templates. This indicates that the product could be related to the presence of excess primer in the initial PCR cycles. The expected size of a product formed by the simple duplication of the *gfp* sequence does not correlate with a product of size 1200bp. The origin of this PCR product, however, remains unclear.

20

Example 4

PNAB2-reverse blocks the formation of rDNA PCR product in rRNA-targeted RT-PCR.

25

rDNA targetted PCR using primers PRBA338F and 511R2, and blocking probe PNAB2-reverse was optimised using pre-primer-annealing temperatures between 56°C-65°C, and annealing temperatures between 45°C-55°C. Optimal PCR protocol (see above) resulted in near-optimal PCR-product formation in the absence of PNAB2-reverse and no visible
30 product in the presence of PNAB2-reverse using genomic DNA from *E. coli* SP308/pKJK10 as template. Using purified PCR-product as template resulted in comparable amounts of PCR-products although PCR was slightly enhanced by the presence of PNAB2-reverse (data not shown). Thus the initial performance of this primer/probe pair was even more promising than PNAB1/*gfpmb1*-Fx. This probably reflect the freedom of choice on where to
35 target the primer/probe pair, avoiding highly structured sequences and allowing a perfect match between probe and target sequence. As we did not get any visible PCR-product in the presence of the probe, construction of a new internal standard to test the extent of inhibition seemed inappropriate, and we chose to test the system directly on RNA samples.

Duplicate samples of total RNA was extracted from approx. 10^7 exponentially growing and from approx. 10^7 starving *E. coli* SP308/pKJK10 cells. One sample was reverse transcribed immediately and the other treated with DNase prior to reverse transcription. Reverse transcription was performed with and without the RT-enzyme and the following PCR was performed with and without the PNAB2-reverse probe. Results are shown in figure 7 (growing cells (a), and starving cells (b)).

Contaminating rDNA was present in both RNA extractions (lane 7 a, b) and DNase treatment efficiently removed contaminating DNA (lane 3 and 4 a, b). The PCR amplification of the contaminating rDNA was efficiently blocked by PNAB2-reverse (lane 8 a, b). Presence of rRNA in the starved cells was not detected after treatment of RNA samples with DNase. However the DNase-treatment resulted in a large "smear" of long PCR-products (lane 1, 2 b). Using PNAB2-reverse and omitting the DNase step showed that rRNA was present in small amounts in the samples from starved cells (lane 6 b). This experiment proves that using this technique has some advantages compared to conventional DNase treatment. Though using a reverse-targeted probe limits the use of the technique to a two-step RT-PCR procedure it seems evident that the increased freedom of choice in primer/probe design adds both efficiency and generality to the developed technique.

20

Example 5

PNAINvA-reverse-1 blocks the formation of DNA based PCR product in quantification of mRNA from invA gene in *Salmonella thyphimorium*

25

rDNA targeted PCR using primers invAF2 and invAR1, and blocking probe PNAINvA-reverse-1 was optimised using pre-primer-annealing temperatures between 62°C-68°C, and annealing temperatures between 52°C-67°C. Optimal PCR protocol (see above) resulted in near-optimal PCR-product formation in the absence of PNAINvA-reverse-1 and no visible product in the presence of PNAINvA-reverse-1 using genomic DNA from *S. thyphimorium* as template. In figure 5 Real Time RT-PCR amplification of cDNA from the *invA* gene of *S. thyphimorium* without PCR signal from the corresponding DNA was accomplished using the PNA inhibitor probe PNAINvA-reverse-1 (compare lane 1 and lane 4). Comparable threshold values are seen for the Real Time analysis of the triplicate samples from DNase treated samples with or without the addition of PNA probe (Table 3). This result shows that the probe does not inhibit the PCR process when cDNA is the template.

Table 3:

Threshold (C_T) values for Real Time PCR amplification of *Salmonella thyphimorium* InvA gene. For an explanation of the C_T -parameter see the previous detailed description of the invention in relation to the real-time PCR technology.

5

Threshold (C_T) values in	With PNA	With PNA	With PNA	No PNA	No PNA	No PNA
Real time PCR	Rep. 1	Rep. 2	Rep. 3	Rep. 1	Rep. 2	Rep. 3
Direct nucleic acid extract (RNA and DNA)	N/A	N/A	N/A	26.9	26.9	27.9
Reverse transcribed after DNA'se treatment	29.3	29.7	28.9	28.5	28.8	29.7
Reverse transcribed no DNA'se treatment	29.5	29.9	28.9	28.8	29.2	28.7

10 REFERENCES

1. **Chen, J. & Griffiths, M.W.** Detection of *Salmonella* and Shiga-like toxin-producing *Escherichia coli* using the magnetic capture hybridization polymerase chain reaction. *Lett. Appl. Microbiol.*, 2001, Vol 32, pp. 7-11

15

2. **Malonrny, B.; Hoorfar, J.; Bunge, C. & Helmuth, R.** Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. *Appl. Environ. Microbiol.* 2003, Vol 69, pp. 290-296.

20 3. **Hunziker, J. and Leumann, C.** (1995) Nucleic Acid Analogues: Synthesis and Properties. In *Modern Synthetic Methods*, Vol. 7, pp. 331-417

4. **Freier, S. M. & Altmann, K. H.** The ups and downs of nucleic acid duplex stability: Structure-stability studies on chemically modified DNA:RNA duplexes. *Nucl. Acid Res.*,

25 1997, 25, 4429-4443

5. **Uhlmann, E.** Recent advances in the medicinal chemistry of antisense oligonucleotides.

Curr. Opin in Drug & Development (2000, 3(2): 293-213.

6. **del Mar Lleo M., S. Pierobon, M.C. Tafi, C. Signoretto, P. Canepari.** 2000. mRNA detection by reverse transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but nonculturable population maintained in a laboratory microcosm. *Appl Environ Microbiol* **66**: 4564-7.
7. **Meckenstock R., P. Steinle, J.R. van der Meer, and M. Snozzi.** 1998. Quantification of bacterial mRNA involved in degradation of 1,2,4-trichlorobenzene by *Pseudomonas* sp. Strain P51 from liquid culture and from river sediment by reverse transcriptase PCR (RT/PCR). *FEMS Microbiol Lett* **167**: 123-129.
8. **Wilson M.S., C. Bakermans, and E.L. Madsen.** 1999. In situ, real-time catabolic gene expression: Extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. *Appl Environ Microbiol* **65**: 80-87.
9. **Alm E.V., and D.A. Stahl.** 2000. Critical factors influencing the recovery and integrity of rRNA extracted from environmental samples: use of an optimized protocol to measure depth-related biomass distribution in freshwater sediments. *J Microbiol Methods* **40**: 153-162.
10. **Tebbe C.C., W. Vahjen.** 1993. Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl Environ Microbiol* **59**: 2657-65.
11. **Anonymous.** 2000. Ambion Technotes Newsletter **7**: 1-3.
12. **Ørum H., P. E. Nielsen, M. Engholm, R. H. Berg, O. Buchardt and C. Stanley.** 1993. Single base pair mutation analysis by PNA directed PCR clamping. *Nucleic Acids Res* **21**: 5332-5336.
13. **Giesen U., W. Kleider, C. Berding, A. Geiger, H. Orum, P.E. Nielsen.** 1998. A formula for thermal stability (T_m) prediction of PNA/DNA duplexes. *Nucleic Acids Res* **26**: 5004-6.
14. **Cochet O., E. Martin, W.H. Fridman., J.L. Teillaud.** 1999. Selective PCR amplification of functional immunoglobulin light chain from hybridoma containing the aberrant MOPC 21-derived V kappa by PNA-mediated PCR clamping. *Biotechniques* **26**: 818-20, 822.

15. **Thiede C., E. Bayerdorffer, R. Blasczyk, B. Wittig, A. Neubauer.** 1996. Simple and sensitive detection of mutations in the ras proto-oncogenes using PNA-mediated PCR clamping. *Nucleic Acids Res* **24**: :983-4
- 5
16. **von Wintzingerode F., O. Landt, A. Ehrlich, U.B. Gobel.** 2000. Peptide nucleic acid-mediated PCR clamping as a useful supplement in the determination of microbial diversity. *Appl Environ Microbiol* **66**: 549-57.
- 10 17. **Sengeløv G., K.J. Kristensen, A.H. Sørensen, N. Kroer, S.J. Sørensen.** 2001. Effect of genomic location on horizontal transfer of a recombinant gene cassette between pseudomonas strains in the rhizosphere and sphermosphere of barley seedlings. *Curr Microbiol* **42**: 160-167.
- 15 18. **Tolker-Nielsen T., U.C. Brinch, P.C. Ragas, J.B. Andersen, C.S. Jacobsen, S. Molin.** 2000. Development and dynamics of *Pseudomonas* sp. biofilms. *J Bacteriol* **182**: 6482-9.
17. **Andersen J.B., C. Sternberg, L.K. Poulsen, S.P. Bjorn, M. Givskov, S. Molin.**
20 1998. New unstable variants of green fluorescent protein for studies of transient gene expression in bacteria. *Appl Environ Microbiol* 1998 **64**: 2240-6
18. **Lanzer M., and H. Bujard.** 1988. Promoters largely determine the efficiency of repressor action. *Proc Natl Acad Sci U S A* **85**: 8973-7.
- 25
19. **Kiyohara H., N. Takizawa, and K. Nagao.** 1992. Natural distribution of bacteria metabolizing many kinds of polycyclic aromatic hydrocarbons. *J Ferment Bioeng* **74**: 49-51
- 30 20. **Kiyohara H., S. Torigoe, N. Kaida, T. Asaki, T. Iida, H. Hayashi, N. Takizawa.** 1994. Cloning and characterization of a chromosomal gene cluster, pah, that encodes the upper pathway for phenanthrene and naphthalene utilization by *Pseudomonas putida* OUS82. *J Bacteriol* **176**. 2439-43
- 35 21. **Bender M., U.C. Brinch, and C.S. Jacobsen.** 2003 Development and application of a quantitative PCR technique targeting *gfp*-tagged flagella mutants of *Pseudomonas putida* OUS82 in soil and sediment columns. **In prep.**

22. **Sturzenbaum S.R.** 1999. Transfer RNA reduces the formation of primer artifacts during quantitative PCR. *Biotechniques* **27**: 50-2.

CLAIMS

1. A method for detecting the presence or absence of at least one target nucleic acid sequence in a sample that further contains a nucleic acid molecule comprising a sequence
5 corresponding to the target nucleic acid sequence, the method comprising the step of contacting the sample with at least one nucleic acid probe that is capable of selectively binding to a continuum of at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence.
- 10 2. A method according to claim 1 further comprising contacting the sample with a first extendable primer.
3. A method according to any of claims 1 and 2, wherein binding of the at least one nucleic
15 acid probe to the nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence prevents annealing of said extendable primer hereto and/or extension of the primer.
4. A method according to any of the preceding claims, wherein the continuum of at least a
20 part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence comprises a transcription initiation site and its upstream sequence.
5. A method according to any of claims 2 to 4, wherein the first extendable primer and a
25 part of the nucleic acid probe are both complementary to a 3' region within said part of the nucleic acid molecule corresponding to the target nucleic acid sequence.
6. A method according to any of claims 2 to 5, wherein the extendable primer has at least one mismatch relative to its complementary sequence in the target nucleic acid sequence.
- 30 7. A method according to any of claims 2 to 6, wherein the extendable primer contains a 3' tag sequence.
8. A method according to any of the preceding claims, wherein the nucleic acid probe
35 comprises a first part, which is complementary to a 3' region within said part of the nucleic acid molecule corresponding to the target nucleic acid sequence, and a second part, which is complementary to a neighbouring sequence of the nucleic acid molecule.

9. A method according to claim 8, wherein said first part of the nucleic acid probe and the first extendable primer are of equal length.
10. A method according to any of claims 8 and 9, wherein said first part of the nucleic acid
5 probe is shorter than the first extendable primer.
11. A method according to any of claims 2 to 10, wherein the T_m -value for the expected hybrid of the nucleic acid probe and the nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence is higher than the T_m -value for the
10 expected hybrid of the first extendable primer and the target nucleic acid sequence.
12. A method according to any of claims 2 to 11, wherein annealing of the nucleic acid probe and annealing of the first extendable primer occur in two successive steps.
- 15 13. A method according to claim 12, wherein the step in which annealing of the nucleic acid probe occurs precedes the step in which annealing of the first extendable primer occurs.
14. A method according to any of claims 2 to 13, wherein the nucleic acid probe is present
20 in molar excess as compared to the first extendable primer.
15. A method according to any of claims 2 to 14, wherein the nucleic acid probe is a nucleic acid analog.
- 25 16. A method according to any of the preceding claims, wherein the nucleic acid probe is a peptide nucleic acid (PNA).
17. A method according to any of claims 1 to 15, wherein the nucleic acid probe is a locked nucleic acid (LNA).
30
18. A method according to any of the preceding claims wherein the nucleic acid probe has at least one mismatch in its first part relative to its complementary sequence in the target nucleic acid sequence.
- 35 19. A method according to any of the preceding claims, wherein the target nucleic acid sequence is one of two complementary nucleic acid sequences.
20. A method according to any of the preceding claims, wherein the target nucleic acid sequence is one of either strand of a double stranded nucleic acid sequence.

21. A method according to any of the preceding claims, wherein the target nucleic acid sequence is a DNA sequence.

5 22. A method according to any of the preceding claims, wherein the target nucleic acid sequence is an RNA sequence.

23. A method according to any of claims 1 to 21, wherein the target nucleic acid sequence is derived from an RNA sequence.

10

24. A method according to any of claims 1 to 21 and 23, wherein the target nucleic acid sequence is a cDNA sequence, such as the sequence of either strand of a double stranded cDNA.

15 25. A method according to any of claims 1 to 21 and 23 and 24, wherein the target nucleic acid sequence is derived from a ribosomal RNA.

26. A method according to any of claims 1 to 21 and 23 to 25, wherein the target nucleic acid sequence is derived from 16S ribosomal RNA.

20

27. A method according to any of claims 1 to 21 and 23 and 24, wherein the target nucleic acid is derived from a messenger RNA.

28. A method according to claim 27, wherein the target nucleic acid sequence is derived
25 from a bacterial messenger RNA.

29. A method according to any of the preceding claims, wherein the target nucleic acid is identical to a nucleic acid sequence, which is specific for one or more microbial species, or its complementary sequence.

30 30. A method according to any of the preceding claims, wherein the target nucleic acid is identical to a nucleic acid sequence, which is conserved among several microbial species, or its complementary sequence.

31. A method according to claims 29 or 30, wherein said microbial species comprise one or
35 more pathogenic species.

32. A method according to any of claims 19 to 24 and 27 to 31, wherein the target nucleic acid sequence is identical to a nucleic acid sequence which is derived from the *invA* gene of *Salmonella Spp.*, or identical to the complementary sequence of said derived sequence

33. A method according to any of claims 19 to 24 and 27 to 30, wherein the target nucleic acid is identical to a the nucleic acid sequence derived from a gene, the product of which is involved in the degradation or conversion of an undesired molecular species, or identical to
5 the complementary sequence of said derived sequence.

34. A method according to any of claims 19 to 24 and 27 to 30, wherein said gene product is involved in the degradation or conversion of a pesticide.

10 35. A method according to any of the preceding claims, wherein the nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence is a genomic DNA molecule.

15 36. A method according to any of the preceding claims, wherein the nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence is a nucleic acid molecule comprising a sequence, which is complementary to the target nucleic acid sequence.

20 37. A method according to any of claims 2 to 36 further comprising contacting the sample with a second extendable primer which is complementary to a part of the complementary strand of said target nucleic acid sequence and detecting the target nucleic acid sequence subsequent to its amplification.

25 38. A method according to claim 37, wherein the T_m -value for the expected hybrid of the second extendable primer and the complementary strand of said target nucleotide sequence is comparable to the T_m -value for the expected hybrid of the nucleic acid probe and the nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence.

30 39. A method according to any of claims 2 to 38, wherein said target nucleotide sequence and its complementary strand is amplified in at least one thermocycle comprising the steps of primer annealing, primer extension and denaturation.

35 40. A method according to claim 39, wherein the amplification occurs in a PCR or in an LCR procedure.

41. A method according to any of claims 39 and 40, wherein the amplification of a target nucleic acid sequence takes place as an integrated part of a RT-PCR procedure.

42. A method according to any of claims 22 to 41, wherein the sample is contacted with the nucleic acid probe prior to reverse transcription of the RNA.

43. A method according to any of claims 37 to 42, wherein the detection is performed
5 using a real time PCR system.

44. A method according to any of the preceding claims further comprising steps of determining the quantity of a target nucleotide sequence in a plurality of nucleic acid molecules
10

45. A method according to any of claims 2 to 44, wherein said first extendable primer is complementary to a nucleotide sequence which is specific for one or more microbial species.

15 46. A method according to any of claims 2 to 45, wherein said first extendable primer is complementary to a nucleotide sequence of a gene the product of which confers resistance towards one or more antibiotics.

47. A method according to any of claims 2 to 31 and 33 to 46, wherein said first
20 extendable primer is complementary to a nucleotide sequence of a gene encoding a microbial toxin.

48. A method according to any of claims 1 to 47, wherein said sample is obtained from a mammal.
25

49. A method according to any of claims 1 to 47, wherein said sample is obtained from a food source.

50. A method according to any of claims 1 to 47, wherein said sample is obtained from a
30 source of drinking water.

51. A method according to any of claims 29 to 50, wherein the microbial species is a bacterial species.

35 52. A method according to any of claims 29 to 51, wherein said microbial species are bacterial species selected from the group consisting of: *Salmonella spp.*, *Escherichia spp.*, *Listeria spp.*, *Shigella spp.*, *Legionella spp.*, *Campylobacter spp.*, *Staphylococcus spp.*, *Clostridium spp.*, *Streptococcus spp.*, *Vibrio spp.*, *Bacillus spp.*, *Yersenia spp.*, *Mycobacterium spp.*

53. A method according to any of claims 29 to 31, 35 to 46 and 48 to 50, wherein the microbial species is a virus.

5 54. A method according to claim 53, wherein the virus is a RNA replicating virus.

55. A method according to any of claims 53 and 54 wherein the virus is a RNA retroid virus.

10 56. A method according to any of the preceding claims further comprising the step of contacting the sample with at least one second nucleic acid probe capable of binding to a continuum of at least a part of the nucleic acid molecule corresponding to a sequence complementary to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence.

15

57. A method according to claim 56 wherein the at least one second nucleic acid probe comprises a first part, which is complementary to a 3' region within said part of the nucleic acid molecule corresponding to a sequence complementary to the target nucleic acid sequence and a second part, which is complementary to a neighbouring sequence of the
20 nucleic acid molecule.

58. A composition for use in the detection of the presence or absence of at least one target nucleic acid sequence in a sample that contains a nucleic acid molecule comprising a sequence corresponding to the target nucleic acid sequence, said composition comprising
25 at least one nucleic acid probe that is capable of selectively binding to a continuum of at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence.

59. A composition according to claim 58 further comprising at least one member selected
30 from the group consisting of polymerases, nucleotides, buffers, salts and stabilising agents.

60. A kit for detecting the presence or absence of at least one target nucleic acid sequence in a sample that contains a nucleic acid molecule comprising a sequence corresponding to
35 the target nucleic acid sequence, said kit comprising at least one nucleic acid probe that is capable of selectively binding to a continuum of at least a part of the nucleic acid molecule corresponding to the target nucleic acid sequence and a part of the nucleic acid molecule adjacent to said corresponding sequence.

61. A kit according to claim 60 further comprising at least one member selected from the group consisting of polymerases, nucleotides, buffers, salts, stabilising agents, and agents for detection of nucleic acids.

1/8

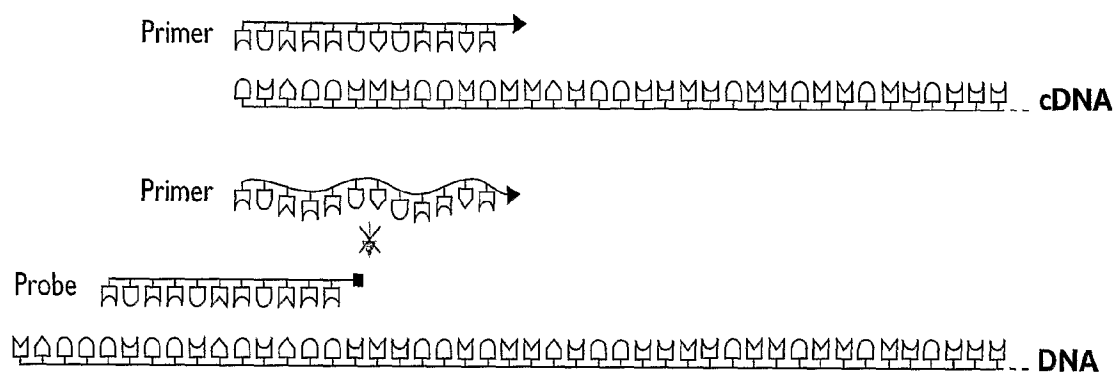


Fig. 1

2/8

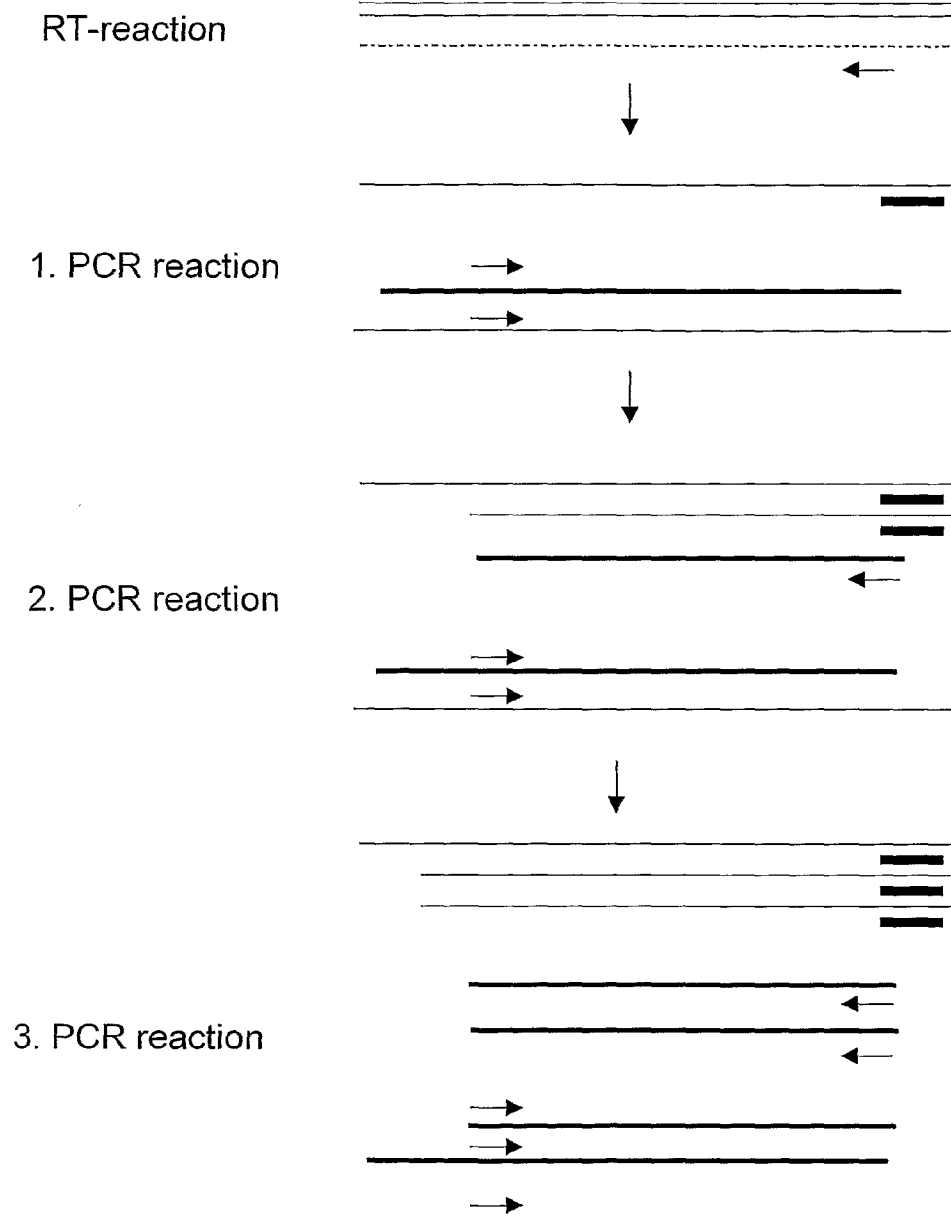
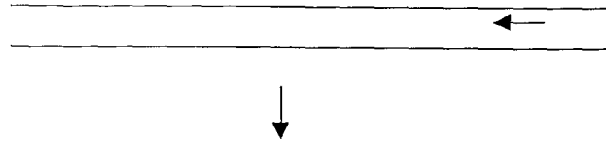


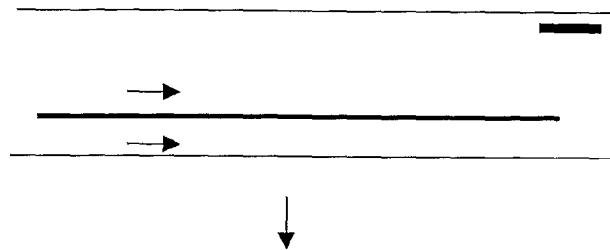
Fig. 2

3/8

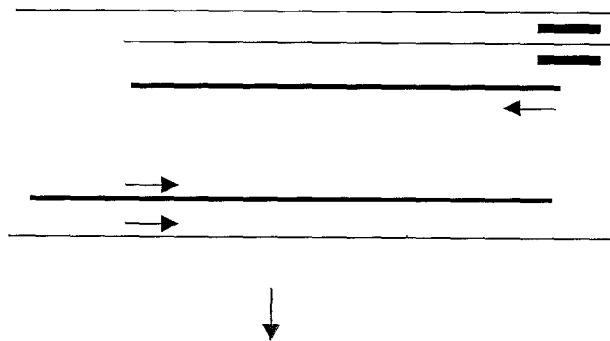
1. PCR reaction



2. PCR reaction



3. PCR reaction



4. PCR reaction

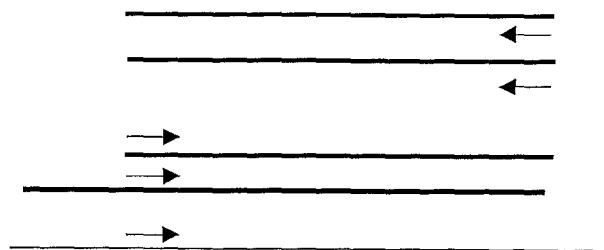


Fig. 3

4/8

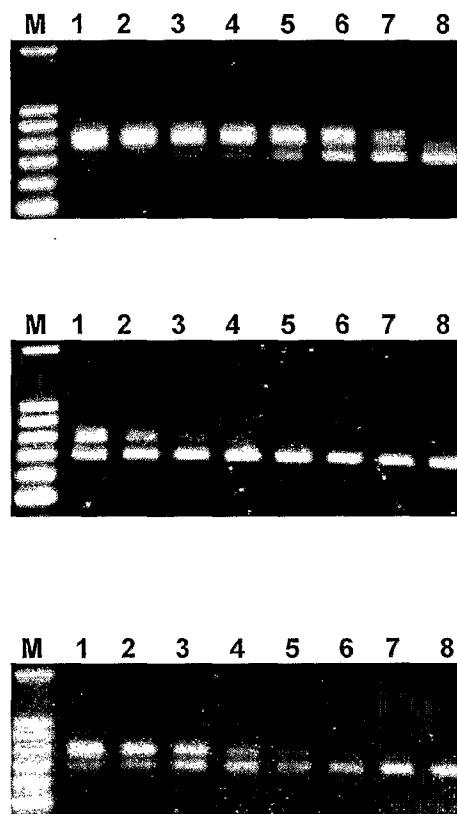


Fig. 4

5/8

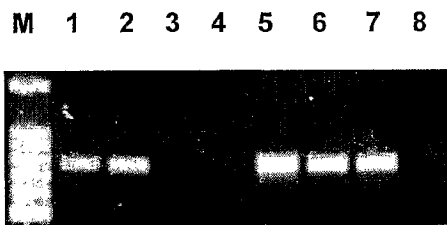


Fig. 5

6/8

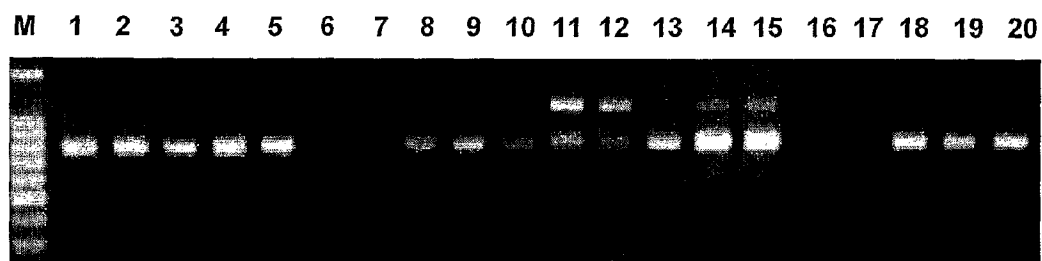


Fig.6

7/8

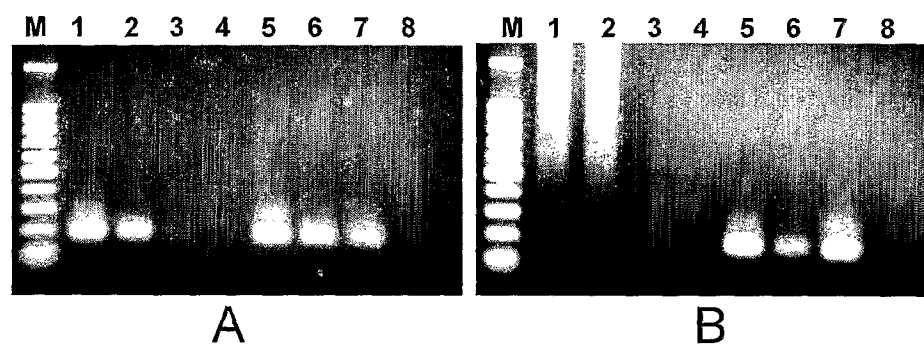


Fig. 7

8/8

1 2 3 4 5 6 M -

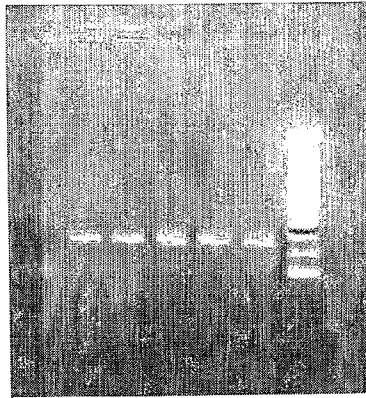


Fig. 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000480

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data, PAJ, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 01/20041 A (MARMARO JEFFREY M ; GERDES JOHN C (US); XTRANA INC (US)) 22 March 2001 (2001-03-22)</p> <p>abstract page 8, line 19 - line 24 page 11, line 6 - page 12, line 3 page 12, line 16 - line 26 page 19, line 10 - line 20; claims 3-11; figures 2,3,11,13; examples 1,6,7; tables I,II</p> <p>----- -/--</p>	<p>1,2,7, 10,11, 21,29, 31, 36-40, 44,45, 48-51, 53-55, 58-61</p>

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

* & * document member of the same patent family

Date of the actual completion of the international search

4 November 2004

Date of mailing of the international search report

19/11/2004

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Tilkorn, A-C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/DK2004/000480

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>US 5 738 993 A (FUGONO NOBUTAKE ET AL) 14 April 1998 (1998-04-14)</p> <p>abstract column 1, line 63 - column 2, line 26 column 4, line 29 - line 41 column 3, line 12 - line 17 column 5, line 44 - column 6, line 22; figure 2</p>	<p>1,18,21, 35,36, 48,58</p>
X	<p>GOOD ET AL: "Antisense inhibition of gene expression in bacteria by PNA targeted to mRNA" NATURE BIOTECHNOLOGY, NATURE PUBLISHING, US, vol. 16, April 1998 (1998-04), pages 355-358, XP002140166 ISSN: 1087-0156 abstract page 355, column 1, paragraph 1 - page 356, column 2, paragraph 1 page 358, column 1, paragraph 3 - column 2, paragraph 1 page 356, column 2, paragraph 4; figures 1-3</p>	<p>1,3,4,8, 16,18, 22-34, 45-47, 51,52, 56-61</p>
X	<p>OERUM H: "PCR CLAMPING" CURRENT ISSUES IN MOLECULAR BIOLOGY, CAISTER ACADEMIC, WYMONDHAM, GB, vol. 2, no. 1, 2000, pages 27-30, XP008014249 ISSN: 1467-3037 abstract page 27, column 2, paragraph 1 - page 28, column 2, paragraph 6 page 29, column 2, paragraph 3 - page 30, column 1, paragraph 2; figures 1-3</p>	<p>1-3,5,6, 9-16, 18-21, 35-40, 43,48, 58-61</p>
A	<p>US 5 849 497 A (STEINMAN CHARLES) 15 December 1998 (1998-12-15) the whole document</p>	<p>1-61</p>
A	<p>WO 98/24933 A (DAKO AS ; GODTFREDSEN SVEN E (DK); BOSTON PROBES INC (US); HYLDIG NIEL) 11 June 1998 (1998-06-11) the whole document</p>	<p>1-61</p>
A	<p>NIELSEN P E ET AL: "SEQUENCE-SPECIFIC TRANSCRIPTION ARREST BY PEPTIDE NUCLEIC ACID BOUND TO THE DNA TEMPLATE STRAND" GENE, ELSEVIER BIOMEDICAL PRESS. AMSTERDAM, NL, vol. 149, 1994, pages 139-145, XP002022886 ISSN: 0378-1119 the whole document</p>	<p>1-61</p>
	-/--	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/DK2004/000480

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 03/020902 A (DATASCOPE INVEST CORP ; ZAK ARIEH S (US); GETTS ROBERT C (US); KADUSHI) 13 March 2003 (2003-03-13) the whole document	1-61
A	<p>-----</p> <p>KYGER ERICH M ET AL: "Detection of hereditary hemochromatosis gene mutation by real-time fluorescence polymerase chain reaction and peptide nucleic acid clamping" ANALYTICAL BIOCHEMISTRY, vol. 260, no. 2, 1 July 1998 (1998-07-01), pages 142-148, XP002303784 ISSN: 0003-2697 the whole document</p> <p>-----</p>	1-61

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/DK2004/000480

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 0120041	A	22-03-2001	US 6258543 B1 AU 1070501 A EP 1224330 A1 WO 0120041 A1	10-07-2001 17-04-2001 24-07-2002 22-03-2001
US 5738993	A	14-04-1998	DE 69516325 D1 DE 69516325 T2 EP 0668361 A1 JP 8070900 A	25-05-2000 18-01-2001 23-08-1995 19-03-1996
US 5849497	A	15-12-1998	NONE	
WO 9824933	A	11-06-1998	US 6110676 A AU 7625998 A DE 69706315 D1 DE 69706315 T2 DK 946750 T3 EP 0946750 A1 WO 9824933 A1 US 2003124521 A1 US 6386113 B1 US 6352032 B1	29-08-2000 29-06-1998 27-09-2001 08-05-2002 17-12-2001 06-10-1999 11-06-1998 03-07-2003 14-05-2002 05-03-2002
WO 03020902	A	13-03-2003	EP 1438435 A2 WO 03020902 A2 US 2004009487 A1	21-07-2004 13-03-2003 15-01-2004